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Observations on a Rapidly Migrating Electrophoretic Component of Cerebrospinal Fluid.*† (18954)

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(Introduced by C. L. Gemmill.)

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Cerebrospinal fluid has been shown to contain an electrophoretic component with a migration rate faster than albumin in 4 of 62 cases studied by Kabat *et al.*(1) and by Scheid and Scheid(2). No evidence was presented by these investigators which would associate a specific disease with this fast component. Evidence is presented in this paper that a rapidly migrating protein is a normal constituent of cerebrospinal fluid.

Methods. Cerebrospinal fluids were obtained from patients undergoing pneumoencephalograms for diagnosis. Fluids collected at the University Hospital were centrifuged immediately and stored in the cold room. Samples collected at the Veterans Hospitals in Richmond and Roanoke were stored in a refrigerator until they were transported to this laboratory in an iced thermos jug. It was determined that storage does not affect the spinal fluid proteins as determined electrophoretically. Fluids showing visible evidence of blood were discarded.

The volumes of the spinal fluid varied between 60 and 200 ml. Each sample was concentrated in the cold room at 4° to a volume of 2-4 ml by positive nitrogen pressure dialysis through collodion sacks(3). The average spinal fluid was concentrated in about 24 hours. This material was dialyzed against a potassium phosphate buffer at pH 7.9 and ionic strength of 0.05.

The electrophoretic technic of Scheid and Scheid(2) was used in a Klett Tiselius apparatus. The lower descending and the horizontal limbs of an 11 ml separation cell were filled with dialyzed plasma. The lower ascending limb was filled with concentrated spinal fluid and the system was filled with the phosphate buffer. The boundary between the spinal fluid and the buffer was sharpened by slowly syphoning buffer through a glass capillary with a right angle tip attached to polyethylene tubing(4,5). Electrophoresis proceeded for one hour at a potential gradient

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† The authors are indebted to the staff members of the University of Virginia Hospital and of the Veterans Hospitals in Richmond and Roanoke for collecting spinal fluids and to Titus Pankey for technical assistance.

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of 3.65 volts/cm. Records were obtained by Longsworth's scanning method(6). Electrophoresis was continued at a potential gradient of 5.48 volts/cm. The albumin boundary was kept stationary by continuous compensation achieved by withdrawing buffer from the upper descending limb through narrow polyethylene tubing. The rate of flow was controlled by adjusting the level of the outflow. After sufficient electrophoresis, the fast component was collected from the upper two-thirds of the ascending limb. These separated samples were immediately frozen and were subsequently thawed, pooled and concentrated by pressure dialysis.

The sedimentation rates were determined in a Model E Spinco ultracentrifuge.

Results. A clearly resolved component of anodic mobility greater than albumin is observed in each of the 65 cerebrospinal fluids studied. This protein, designated as component X, has a mean mobility of 11.8 $\times$ 10^{-5} cm^2 volt^{-1} sec.$^{-1}$, indicating a migration rate 1.3 times faster than albumin. In 9 cases a small component of anodic mobility greater than component X was resolved. This protein, designated as component X_1, has a mean mobility of 14.0 $\times$ 10^{-5} cm^2 volt^{-1} sec.$^{-1}$. Preliminary analyses indicates that component X is resolvable between pH 4.0 and 7.9 and has an isoelectric point between pH 4.0 and 4.5. An electrophoretic pattern showing both components is presented in Fig. 1.

The mean relative concentration of component X in dilute phosphate buffer is approximately 9% of the total protein with individual values varying between 3 and 17%. The true values for this component are probably less since boundary anomalies are appreciable in dilute phosphate buffer. No differences in the concentrations of component X are observed in spinal fluids from patients with schizophrenia, brain tumor or epilepsy. The mean relative concentration of component X_1 is about 1% of the total protein; its presence could not be associated with any particular disease.

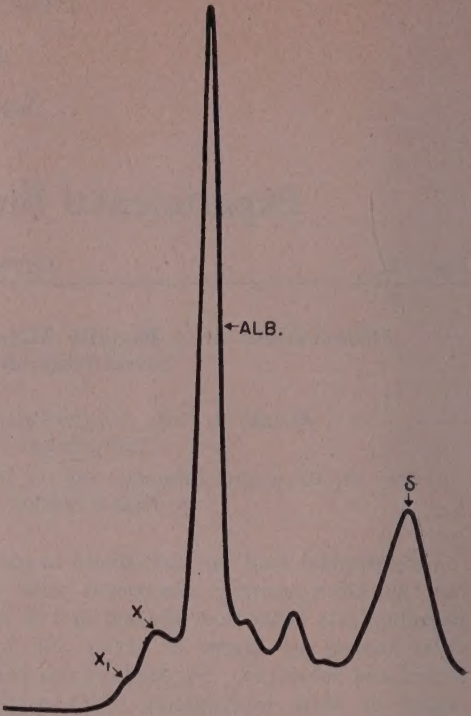

FIG. 1. Electrophoretic pattern of concentrated cerebrospinal fluid.

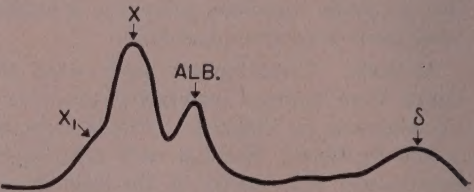

FIG. 2. Electrophoretic pattern of concentrated rapidly migrating components of cerebrospinal fluid.

The separated fast components of 30 spinal fluids were pooled and concentrated by pressure dialysis. The electrophoretic pattern of this concentrated fraction (Fig. 2) shows the presence of 2 main components. The larger component has the mobility of component X and represents 63% of the total area. The asymmetry of the boundary at the leading edge is presumably due to component X_1. The remaining component has the mobility of albumin. Ultracentrifuge analysis of the concentrated pooled fractions indicates that over 95% of the protein sediments as a homogeneous component with the sedimentation

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rate of albumin. The remaining protein sedimented 2.2 times more rapidly than the main component.

Discussion. A rapidly migrating component is readily observed in the electrophoretic patterns of concentrated spinal fluids equilibrated with a phosphate buffer (pH 7.5, $\Gamma/2$ .05) or a veronal buffer (pH 8.6, $\Gamma/2$ 0.1). Kabat *et al.*(1) using a phosphate-sodium chloride buffer mixture at pH 7.4 and an ionic strength of about 0.2, recorded the presence of a fast component in 3 of 40 spinal fluids. Scheid and Scheid(2) studied spinal fluids which were not sufficiently concentrated and observed the fast component in 1 of 22 cases. These studies emphasize the necessity of using concentrated fluids and suitable buffer mixtures if the rapidly migrating components of spinal fluid are to be observed or separated electrophoretically.

Component X has a greater anodic mobility than albumin over a pH range varying from 7.9 to 4.0. In contrast, rapidly migrating components observed in plasma do not behave similarly over this pH range(7). Furthermore, plasma adjusted to the protein concentration of concentrated spinal fluid and equilibrated against dilute phosphate buffer does not reveal the presence of component X. Under these conditions the albumin boundary of plasma spreads appreciably and may prevent the detection of small amounts of the fast component. From the available data, it appears justifiable to assume that components X and X_1 originate in the nervous system and are proteins typical for cerebrospinal fluid.

Summary. Electrophoretic studies of 65 cerebrospinal fluids showed the presence of a rapidly migrating protein in each case. This component migrated about 1.3 times faster than albumin and sedimented at the same rate as albumin in the ultracentrifuge. In 9 cases, 2 rapidly migrating components were observed. Under the experimental conditions used, the fast components comprise about 9% of the total spinal fluid proteins.

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Hydrolysis of Conjugates of Neutral Ketosteroids. (18955)

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The study of the metabolism of steroids is, in part at least, dependent upon the determination of the quantities and types of steroids excreted in the urine. Important information could also be obtained if it were possible to estimate the individual steroid conjugates so excreted.

The usual method of hydrolyzing the steroid conjugates by refluxing strongly acidified urine has been shown to cause alterations of at least some of the steroids(1-9). Furthermore, such treatment would be expected to hydrolyze all of the conjugates to a greater or lesser degree. Numerous attempts have been made to reduce the drastic nature of hot acid hydrolysis by employing simultaneous extraction(10-15) and the milder and more specific method of enzymatic hydrolysis(4, 16-27).

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* Atomic Energy Commission Predoctoral Fellow in the Biological Sciences.

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In a previous publication(21) in which we reported that the β-glucuronidase produced by *E. coli*(28,29) hydrolyzes a large proportion of the estrogen conjugates of human pregnancy urine, we presented data to show that this means of hydrolysis also liberates a very considerable quantity of the neutral ketosteroids from these urines as well as from the urine of normal men. Subsequently, Bitman and Cohen(23) reported the use of ox-spleen β-glucuronidase for the liberation of 17-ketosteroids in urine. The data here described were obtained from a continuation of attempts to develop mild, yet effective methods of hydrolyzing the conjugated steroids and to determine their distribution in urine.

Since neutral ketosteroids are known to be excreted in part in conjugation with sulfuric acid, it would be highly desirable to have available an enzyme capable of hydrolyzing such esters. Our bacterial glucuronidase preparations which are apparently able to split all types of β-glucuronides have no effect on phenolic or alcoholic sulfates. This is also true of spleen glucuronidase(20). Further, the phenolsulfatase present in takadiastase and Mylase P preparations which effectively hydrolyzes estrone sulfate(20) has, in our hands at least, no detectable effect on the sulfates of androsterone and dehydroepiandrosterone.

Attempts to obtain sulfatases of microbiological origin have shown these enzymes to be highly specific, but none has been found to hydrolyze the sulfates of the neutral 17-ketosteroids. We have studied 23 types of bacteria as a possible source of alcoholic sulfatase. Of these only *Proteus vulgaris* and *Pseudomonas non liquefaciens*(30) exhibited sulfatase activity. The growing cultures and the culture fluids of these 2 organisms liberated sulfate from chondroitin sulfate but did not hydrolyze the sulfates of menthol, pentaerythritol, cholic acid, androsterone and dehydroepiandrosterone. The addition of these sulfates to the culture media did not stimulate the production of sulfatase. (Unpublished results).

Experimental. In the studies described below, the method of Holtorf and Koch(31) was used for estimating the androsterone and dehydroepiandrosterone, and estrone was determined by the Kober reaction according to the modification of Salter *et al.*(32). When

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TABLE I. Hydrolysis of Pure Steroid Sulfates. % recovered in form of free steroid as measured by m-dinitrobenzene reaction for ketosteroid and Kober reaction for estrone.

	Method of hydrolysis				
Conjugate	Bitman-Cohen or Talbot, *et al.*	Autoclaving, 15 lb –1 hr pH 7.3	Ether 24 hr pH 0.7	Ether 24 hr 7.2 N HCl	HCl 15 vol %, 10 min
Dehydroepiandrosterone sulfate	90–95	90	92	98	10
Androsterone sulfate	0	0	90	95	25
Estrone sulfate	—	0	95	93	98

hydrolysis of urine or of solutions prepared from butanol extracts of urine was employed, neutral fractions were obtained by the procedure of Friedgood *et al.*(33) and the neutral ketosteroids were estimated after removing the non-ketonic fraction by Girard's reagent according to the procedure of Pincus and Pearlman(3).

Hydrolysis by dilute acid. Dobriner *et al.*(14) have reported the liberation of significant quantities of neutral steroids from urine acidified to pH 1.0 and extracted continuously with ether at room temperature. Lieberman *et al.*(13) have shown that such extraction of solutions acidified to 0.2 N HCl hydrolyzes the sulfates of cholesterol, cholestanol, and dehydroepiandrosterone to the extent of 90-93%.

Talbot *et al.*(4) recovered dehydroepiandrosterone quantitatively from its sulfate by heating for 4 hours at 100°C an aqueous solution of the conjugate buffered with acetate at pH 5.5 and containing 15% $BaCl_2$. Bitman and Cohen(34) have shown that $BaCl_2$ is not necessary for the hydrolysis of this conjugate and that such treatment does not affect the glucuronide linkage(23).

We have found(15) that the methods of Talbot *et al.* and of Bitman and Cohen effectively hydrolyze dehydroepiandrosterone sulfate but do not affect androsterone sulfate to any detectable extent. This behavior is also exhibited when the aqueous solutions of these substances at pH 7.3 are autoclaved at 120°C for 1 hour. The data in Table I show the marked difference between these 17-ketosteroid sulfates when they are subjected to these very mild hydrolytic procedures.

Continuous extraction of the aqueous solutions at pH 0.7 with ether for 24 hours, at the temperature of the boiling point of ether, satisfactorily hydrolyzed the sulfates of both androsterone and dehydroepiandrosterone as well as of estrone (Table I) as measured by the respective colorimetric methods. The glucuronides of estriol, menthol, pregnanediol and phenolphthalein were not affected by this treatment. It, therefore, appears that hydrolysis at pH 0.7 under these conditions may afford a means of distinguishing steroid sulfates from steroid glucuronides.

Hydrolysis by concentrated acid. The conventional procedure for hydrolyzing the steroid conjugates by refluxing with 10-15 volumes % of concentrated HCl for 10 minutes has been shown to be destructive(1-9). The poor recovery which resulted when the aqueous solutions of pure androsterone sulfate and dehydroepiandrosterone sulfate were refluxed with 15 volumes % HCl for 10 minutes confirms this observation (Table I). Longer periods of hydrolysis yielded even lower recoveries. However, estrone is quantitatively recovered from its sulfate by this method (Table I).

Since the steroid glucuronides are not readily hydrolyzed by acid and since the destruction of the neutral 17-ketosteroids by this treatment is probably a function of the temperature and the acid concentration, we have attempted to find a set of conditions which would minimize the destruction and yet permit satisfactory hydrolysis. Using 7.2 N HCl for hydrolysis at the temperature of boiling

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TABLE II. Hydrolysis of Conjugated Ketosteroids of Normal Male Urine. Expressed as mg of dehydroepiandrosterone per 24 hr.

Urine	Method of hydrolysis				
	Bitman-Cohen	Ether 24 hr pH 0.7	Glucuronidase	HCl 15 vol %, 10 min	Ether 24 hr 7.2 N HCl
1	1.9	3.4	6.5	6.7	12.2
2	3.3	5	8.9	10	14.9
3	1.2	1.3	7.5	8.3	10.4
4	1.3	2	10.3	10.7	10.6
5	1.2	2.5	4.6	5.8	7.8
6	2.2	4.3	6.8	7.8	11.6

TABLE III. Hydrolysis of Conjugated Neutral Ketosteroids of Urines of Patients with Adrenal Pathology. Expressed as mg of dehydroepiandrosterone per 24 hr.

Urine No.	Type of case	pH 0.7 ether 24 hr	Glucuronidase	HCl 15 vol %, 10 min	HCl (7.2 N) ether 24 hr
1	♀—33 yr old; adrenal hyperplasia	11.4	25.8	41	46
2	♀—1½ yr old; adrenal tumor	90	8.5	65	93
3	♀—57 yr old; adrenal tumor	90	35	92	118
4	♀—2 mo old; adrenal hyperplasia	6.6	6	16.7	25.5
6	♀—6 yr old; sexually precocious	10	8	18.4	25.5

ether (35°C), androsterone, dehydroepiandrosterone, and estrone as measured by their colorimetric values are practically quantitatively recovered from their sulfates by continuous extraction with ether for 24 hours (Table I). The estrone exhibited little or no loss of biological activity as the result of this treatment. Furthermore, hydrolysis of conjugates in urine by this procedure in all but one instance gave significantly higher ketosteroid values than were obtained by refluxing with 15 volumes % HCl (Tables II and III). However, estriol and pregnanediol were recovered to the extent of only about 60% from aqueous solutions of their glucuronides. An additional 20% of estriol was obtained by treating the bicarbonate washings of the ether phase with glucuronidase. Appreciable amounts of pregnanediol could not be recovered from the ether phase, nor were additional amounts of either steroid obtained from the aqueous phase after reducing the HCl concentration to 15 vol. % and refluxing for 10 minutes. It would thus appear that a considerable destruction of these steroids had occurred. These findings may indicate that the urinary 17-ketosteroid glucuronides also are not completely recovered under these conditions but, owing to the unavailability of pure specimens of this class of compounds, this point has not been directly studied. However, the higher yields obtained by the use of 7.2 N HCl suggest that the ketosteroid glucuronides behave differently than do those of estriol and pregnanediol under these conditions.

Hydrolysis of urinary conjugated neutral ketosteroids. A study was made of the amounts of neutral ketosteroids which were liberated from the urine of normal men and of patients with adrenal pathology by the methods described above as well as by bacterial β-glucuronidase.

The normal urines were kept in the refrigerator during collection; they were then boiled at neutral pH for 10 minutes to destroy enzymes, and after removal of the free steroids with ether, they were preserved with thymol and stored in the refrigerator at 5°C.

Enzymatic hydrolysis was performed by incubating aqueous solutions of the butanol-soluble extractives of urine prepared according to Bitman and Cohen(34) with the bacterial glucuronidase for 24 hours at 38°C and at pH 6.5. An amount of β-glucuronidase capable of liberating approximately 5000 μg of phenolphthalein from phenolphthalein glucuronide in a 4-hour assay at pH 6.2 was used for each 10 ml of urine and $CHCl_3$ was used as the preservative. Butanol extracts instead of whole urine were employed for the enzymatic hydrolysis in order to avoid concentrations of ammonium ion which we had found(29) to have an inhibiting effect on this β-glucuronidase and which had developed in some of the urines during storage even though they were preserved with thymol. The ketosteroid values obtained when fresh whole urines are incubated are the same as those obtained with their butanol extracts. However, upon becoming ammoniacal low values are obtained with the untreated urines while their butanol extracts give the same values as those obtained on fresh urine. We have subsequently employed $CHCl_3$ as the preservative which is much superior in this regard.

The data obtained in our study of the neutral ketosteroids of the urine of normal men are presented in Table II. It can be seen that hydrolysis by the method of Bitman and Cohen liberated a significant quantity of ketosteroid in each instance. However, continuous extraction with ether at pH 0.7 without recovering unhydrolyzed steroid glucuronides from the organic phase according to Dobriner and Lieberman(35) resulted in decidedly higher values in all but one instance. This is not surprising in view of our finding that androsterone sulfate is not hydrolyzed under the conditions of the method of Bitman and Cohen but is effectively hydrolyzed by continuous extraction with ether at pH 0.7, while dehydroepiandrosterone sulfate is hydrolyzed by both procedures. It is quite likely that other neutral ketosteroids behave similarly to androsterone and dehydroepiandrosterone in this regard and that these procedures may be of great importance in enabling one to distinguish between these two types of ketosteroid sulfates. Such a distinction may be of value in steroid metabolism studies.

Hydrolysis of these urines by bacterial β-glucuronidase resulted in the liberation of more neutral ketosteroid than was obtained by continuous ether extraction at pH 0.7. Since the latter procedure apparently does not hydrolyze glucuronides and since our glucuronidase preparations contain no demonstrable sulfatase, it is tempting to assume that hydrolysis with glucuronidase measures the ketosteroid glucuronides while continuous extraction with ether at pH 0.7 gives a measure of the ketosteroid sulfates in these urines. If such an assumption is valid then the quantity of neutral ketosteroids conjugated with glucuronic acid, in every case, exceeded that conjugated with sulfuric acid.

Somewhat higher values were obtained by refluxing the urines for 10 minutes with 15 vol. % of concentrated HCl than by treatment with glucuronidase. However, with one exception, the highest values were obtained by the continuous ether extraction of the urines acidified to 7.2 N HCl. In most instances, these values (total ketosteroid?) approximate the sum of the values obtained by continuous extraction with ether at pH 0.7 (ketosteroid sulfates?) and by glucuronidase hydrolysis (ketosteroid glucuronides?).

The quantity of conjugated ketosteroids which is extracted by ether(35) from urine acidified to 7.2 N HCl was found to be very small. The bicarbonate washings of the ether obtained by treating these normal male urines in this manner, contained only 1-4.7% of the amount of ketosteroid remaining in the ether. This was ascertained both by hydrolysis with glucuronidase and refluxing with 15 vol. % HCl. The ketosteroid extracted by the bicarbonate is apparently conjugated with glucuronic acid for acid hydrolysis following treatment with glucuronidase did not give additional ketosteroid.

Although the amount of conjugated ketosteroid extracted by the ether is small under these conditions, for greater accuracy it may be desirable to treat the bicarbonate washings with glucuronidase.

Although we had shown that androsterone and dehydroepiandrosterone, measured as 17-ketosteroid, are quantitatively recovered from their sulfates in a solution of 7.2 N HCl by continuous extraction with ether at 35°C, the possibility remained that the stability of these substances under these conditions might be altered when they are present in urine. That this is not true is indicated by the recovery by this method of 88-93% of 125 γ dehydroepiandrosterone added as the sulfate to 10 ml

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35. Dobriner, K., and Lieberman, S., *Steroid Hormones* (University of Wisconsin Press, 1950), page 49-50.

portions of 5 normal male urines.

The results obtained from the study of urines of patients with adrenal pathology are shown in Table III. Urines 1-4, in the form of butanol extracts(36), were kindly supplied by Dr. Willard M. Allen of the Department of Gynecology and Obstetrics of Washington University. The diagnosis of each of these cases was found to be correct by appropriate operation. Urine 6, as fresh whole urine, was obtained by Dr. Daniel Sexton of the Department of Medicine of St. Louis University from a case of sexual precocity.

Here again, as with the normal urines, ether extraction after acidification to 7.2 N HCl and removal of the non-ketonic fraction gave neutral ketosteroid values which were higher than those obtained by the other methods of hydrolysis. It is interesting to note that in these adrenal tumor cases in which the total neutral ketosteroid excretion was very high, the fraction hydrolyzed at pH 0.7 greatly exceeded that hydrolyzed by glucuronidase. In the cases of adrenal hyperplasia the total neutral ketosteroid excretion was considerably lower while the fraction hydrolyzable by glucuronidase was relatively larger. It is obvious, of course, that a larger number of cases must be studied before it can be ascertained whether this difference in distribution of neutral ketosteroid conjugates can be used to distinguish between hyperplasia and tumors of the adrenal cortex.

In preliminary studies, we have applied the color test which, according to Allen, Hayward, and Pinto(38) is specific for dehydroepiandrosterone and certain related steroids, to the neutral ketonic fraction of the hydrolysates obtained from these pathological urines. We have confirmed the observation made by these and other investigators(19,37-43) that the urines of the patients with adrenocortical tumors contain large amounts of this ketosteroid.

In addition, we have found that none of this substance was liberated by glucuronidase while a large portion of it was obtained by continuous extraction with ether at pH 0.7. It is possible that these results are related to the presence of i-androstanolone which has been shown by Dingemanse *et al.*(9) to be converted by HCl to dehydroepiandrosterone. Unfortunately, the procedure of Bitman and Cohen was not applied to these urines. Higher values were obtained when 7.2 N HCl was used. Furthermore, the color developed with the extracts following hydrolysis at pH 0.7 and in 7.2 N HCl was much better than that obtained after refluxing with 15 vol. % of concentrated HCl, readily permitting the detection of the much smaller amounts of this ketosteroid in the urines of patients with adrenal hyperplasia.

Summary. The hydrolysis of steroid conjugates has been studied by 5 methods. Continuous ether extraction of urine made 7.2 N with respect to HCl gave values appreciably higher than those obtained by boiling with 15 vol. % HCl and these values approximated the sum of ketosteroids released by β-glucuronidase and by continuous extraction with ether of urines brought to pH 0.7 with HCl.

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Relaxin and Mammary Growth in the Mouse. (18956)

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Daily administration of .83 μg of estradiol benzoate, 1 mg of progesterone, and 25 guinea pig units of relaxin for 13 days produced an advanced degree of mammary alveolar development in ovariectomized immature rats. Combinations of any two of these hormones caused only a slight growth of the mammary gland(1). Since an advanced degree of development of the mammary gland of the castrated male or female rat may be obtained with larger dosages of estrogen and progesterone(2,3), exogenous relaxin, while enhancing the response, is not essential for the mammary alveolar response to estrogen and progesterone. In the guinea pig, in which estrogen alone is capable of producing full alveolar development, a quantitative increase in the degree of development was achieved by the addition of relaxin to the estrogen(4). In the rabbit, addition of relaxin to estrogen transferred the emphasis of growth from the primary ducts to the entire ramifying duct system without inducing significant alveolar growth. These authors conclude that the action of relaxin in respect to mammary growth is in the role of a potentiator of estrogen rather than producing specific changes attributable to relaxin *per se*(4).

The present report represents an attempt to determine the effect of relaxin on mammary growth in the mouse. Mixner and Turner (5,6) developed a method for the assay of materials which stimulate mammary alveolar development in the ovariectomized virgin female mouse. The assay mice receive 10 daily injections of a low level of estrogen, in itself insufficient to cause an alveolar response. When graded doses of progesterone were simultaneously administered, the percentage of mice showing a minimum but definite alveolar response was directly proportional to the dose of progesterone. A total dose of from .75 to 1 mg of progesterone gave a positive response in approximately 50 per cent of the mice. The level of estrogen could be varied over a fairly wide range without significantly altering the response obtained.

Methods and materials. Using mice of different strains than those used by Mixner and Turner, a lower order of response to estrogen and progesterone was observed (Table I). Mixner and Turner (personal communication) have observed strain differences in the mammary response of mice to estrogen and progesterone, 3 additional sources of mice showing a lower response than those originally reported on.

In an attempt to carry the extent of alveolar development beyong the minimal or initial stage, 3 ovariectomized C_{57} mice were given .3 μg of estradiol benzoate plus 1 mg of progesterone daily for 21 days (6.3 μg plus 21 mg total). Each mouse showed only a slight alveolar development. It was felt that perhaps the addition of relaxin might result in a more advanced degree of alveolar response to estrogen and progesterone, as well as in a higher percentage of positive responses. Ovariectomized virgin female mice received either 10 or 20 daily injections of estradiol benzoate, progesterone, and relaxin,† in various combinations, in the dosages indicated in Tables II and III. A mammary biopsy was taken at

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* Fellow of The Jane Coffin Childs Memorial Fund for Medical Research. This investigation has been aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research.

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† The relaxin, generously provided by Dr. R. L. Kroc, Chilcott Laboratories, Morris Plains, N. J., was prepared from sow ovaries and assayed 100 GPU per cc.

TABLE I. Mammary Alveolar Response of Ovariectomized Virgin Female Mice to 10 Daily Injections of Estrogen and Progesterone.

Strain	Total dose		No. of mice	Response	
	Estrone (I.U.)	Progesterone (mg)		Pos.	Neg.
CBA × A Hybrids (F_1)	75	1.	6	0	6
”	75	2.	6	1	5
C_3H	75	2.	9	3	6

TABLE II. Mammary Alveolar Response of Ovariectomized Virgin Female Mice to 10 Daily Injections of 1 μg Estradiol Benzoate, 1 mg Progesterone, and 25 Guinea Pig Units of Relaxin in Various Combinations.

Strain	Treatment	No. of mice	Response		Avg interpubic gap (mm)
			Pos.	Neg.	
C_3H	E*	5	1	4	.5
	E + R	4	1	3	.9
	E + P	4	1	3	0
	E + P + R	4	3	1	.07
C_3H × A Hybrids (F_1)	E + P	12	2	10	.03
” ”	E + P + R	12	4	8	.14

* E = estrogen. R = relaxin. P = progesterone.

TABLE III. Mammary Alveolar Response of Ovariectomized Virgin Female Mice to 20 Daily Injections of 1 μg Estradiol Benzoate, .25 mg Progesterone, and 25 Guinea Pig Units of Relaxin in Various Combinations.

Strain	Treatment	No. of mice	Response		Avg interpubic gap (mm)
			Pos.	Neg.	
C_3H × A and CBA × A Hybrids (F_2 and F_3)	Untreated controls	3	0	3	0
	E*	4	0	4	1.5
	E + R	4	0	4	2.5
	E + P	4	4	0	.4
	E + P + R	4	4	0	1.5

* E = estrogen. R = relaxin. P = progesterone.

the time of ovariectomy to eliminate "false positive" animals. Those mice receiving all 3 hormones for 10 days showed a somewhat higher percentage of positive responses than those receiving only estrogen and progesterone (Table II). In neither case, however, was alveolar development very extensive. Of the mice receiving 20 injections, all those receiving estrogen and progesterone showed a positive response whether or not relaxin was also given (Table III). The extent of alveolar development was roughly comparable in the 20-day estrogen plus progesterone, and estrogen plus progesterone plus relaxin groups, with only one mouse in the latter group showing a more advanced development than in the former group. This animal showed a development equal to that of the 10th or 12th day of pregnancy. It is apparent, therefore, that even the most advanced degree of alveolar development obtained did not approach that of late pregnancy. The mouse appears to differ from the rat with respect to the rate and extent of mammary growth attainable with the ovarian steroids, and with respect to the effect of added relaxin. It is possible that higher levels of relaxin may be required in the mouse than in the rat, or that dividing the daily dose of relaxin, as was done by Hamolsky and Sparrow, may give a greater effect.

Measurements of the interpubic gap (Tables II and III) provide confirmation of the observation of Hall(7) that in the mouse, unlike the guinea pig, progesterone inhibits the pelvic relaxing effect of estrogen or of estrogen plus relaxin.

A composite x-ray photograph was taken of one femur from each of 16 of the 19 mice on the 20-day experiment. The expected increase in bone density of the estrogen-treated

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mice, as compared to the untreated controls, was observed(8). No significant modifying effect of either progesterone or relaxin was apparent.

Summary. Little or no increase in the percentage of positive mammary alveolar responses was obtained in ovariectomized mice treated with estrogen plus progesterone plus relaxin as compared to those treated with estrogen plus progesterone only. Progesterone inhibited the pelvic relaxing effect of estrogen or of estrogen plus relaxin. Neither progesterone nor relaxin exerted any significant modifying effect on the increased bone density resulting from estrogen administration.

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Protection of Mice Against Vaccinia Virus by Administration of Benzaldehyde Thiosemicarbazone.* (18957)

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Domagk and associates(1) reported that benzaldehyde thiosemicarbazone inhibited growth of the tuberculosis bacillus *in vitro*. Certain derivatives of this compound were found to protect experimental animals against tuberculosis(2). Hamre *et al.*(3,4) investigated the effect of substituted benzaldehyde thiosemicarbazones on other microorganisms. The *p*-amino analogue was observed to delay death and to protect a small percentage of chick embryos and mice infected with vaccinia virus. The *p*-acetamido analogue was less effective.

The present report deals with the effect of benzaldehyde thiosemicarbazone and of several *para* substituted derivatives on vaccinia virus. The compounds used in the study were obtained from Dr. A. F. Langlykke of E. R. Squibb and Sons.

Experimental. In *in vitro* experiments the CVII strain of vaccinia virus was cultivated in a medium containing minced chick embryonic tissues(5). The International Health Division (IHD) strain of virus was employed in *in vivo* tests(6). Mice, in groups of 6, were inoculated with viral dilutions of 10^{-2}, 10^{-3} and 10^{-4} by the cerebral route. Materials to be tested were fed to mice in a sucrose-casein diet.

Results. Benzaldehyde thiosemicarbazone (No. 1) was found to inhibit completely viral multiplication when present in a concentration of 1 μg per ml of culture medium (Table I). Substitutions in the *para* position of the benzene nucleus reduced activity in all instances (No. 2-8). The introduction of an isobutyl group in the 4-position of the thiosemicarbazone portion of the molecule (No. 9) likewise decreased *in vitro* activity. With the exception of compound No. 3, none of the substances was completely in solution when added to the culture medium.

The failure of vaccinia virus to multiply in the presence of thiosemicarbazones does not appear to be due to inactivation of the virus by these substances. Benzaldehyde thiosemicarbazone was added to Tyrode's solution containing 5% normal rabbit serum and a known strength of vaccinia virus to give a concentration of 0.05 mg of the compound per ml of

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TABLE I. Effect of Benzaldehyde Thiosemicarbazones on Vaccinia Virus.

No.	Substituent in *p*-position of benzene ring	Conc. (mg/ml)	Inhibition of viral growth in chick embryonic tissues change in titer of virus (log)		Protection of mice against virus (Intracerebral)				
			In presence of compound	In absence of compound	Conc. in diet (%)	Treatment started (day)	Proportion of mice surviving infection: Treated	Proportion of mice surviving infection: Untreated	Excess survivors in treated groups
	Benzaldehyde thiosemicarbazones								
1	—	.1	−2 *	1.9	.02	−2	10/16	2/16	8
		.01	−1.6*	1.5	.04	−2	11/17	2/16	9
					.08	−2	10/15	2/16	8
		.001	−2.2*	1.5					
					.03	−3	8/18	4/18	4
					.04	−2	9/18	4/18	5
					.04	−2	13/18	2/18	11
					.04	0	10/18	2/18	8
					.04	2	7/17	2/17	5
					.04	2	6/18	2/18	4
					.04	−2	9/18	1/17	8
					.04	−2	12/18	3/18	9
					.04	−2	9/17	1/17	8
					.04	−1	13/18	6/18	7
					.03	−3	17/18†	7/18†	10
2	hydroxy	.05	− .5	1.9	.04	−2	5/17	5/18	0
					.04	−1	6/18	3/18	3
3	amino	.1	− .7	1.9	.04	−2	4/15	2/17	2
					.04	−1	2/17	1/17	1
					.04	0	7/17	5/17	2
4	acetamido	.1	.1	1.9	.04	0	5/18	1/18	4
					.05	−2	3/17	6/18	−3
					.06	−3	0/18	4/18	−4
					.1	0	2/17	1/18	1
5	dimethylamino	.05	− .3	1	.04	−3	5/18	6/18	−1
6	isopropyl	.05	−2 *	1	.04	−3	1/18	3/18	−2
		.005	.7	1.3					
7	ethylsulfonyl	.05	−1.3*	1.9	.04	−1	5/17	4/18	1
		.01	1	1.5	.04	−1	10/18	5/18	5
					.06	−2	3/18	3/18	0
8	isobutoxy	.05	−2.3*	1.4	.025	−2	5/18	3/18	2
		.005	−1	1.5	.04	−2	7/18	3/18	4
					.04	−1	4/18	1/18	3
					.04	1	5/18	2/17	3
	Benzaldehyde 4-isobutyl thiosemicarbazones								
9	—	.05	− .5	1	.04	−3	5/18	6/18	−1
10	acetamido	.05	− .5	1.4	.04	−2	2/18	3/18	−1
11	dimethylamino	.05	−1	1	.04	−3	5/18	6/18	−1
12	ethylsulfonyl	.05	2.3	1.9	.04	−3	6/18	6/18	0

* Or greater.

† Mice were inoculated with the WR strain of virus; in all other experiments the IHD strain was used.

medium. After incubation at 32°C for two days the concentration of virus was found to have decreased 2.3 log units. In corresponding control tests the reduction was 1.9 log units. In a comparable experiment with the *p*-acetamido analogue, using a concentration of 0.1 mg per ml of medium, reductions of 2.8 and 2.2 log units, respectively, were obtained.

In *in vivo* tests, benzaldehyde thiosemicarbazone was found to be more active than any other member of the series (Table I). In 14 experiments in which this compound was fed in the diet in concentrations varying from 0.02 to 0.08%, 144 of 244 treated mice (59%) survived in contrast to 40 of 243 untreated animals (16%). Treated mice which succumbed to infection died on the average of one day later than the corresponding controls. In one experiment benzaldehyde thiosemicarbazone was used for the treatment of mice inoculated intracerebrally with the WR

(Western Reserve) strain of vaccinia virus. In this experiment, 17 of 18 treated mice survived as compared to 7 of 18 untreated animals.

The number of survivors in treated groups exceeded the number in control groups in experiments with *p*-amino (3 of 3), *p*-isobutoxy (4 of 4), *p*-ethylsulfonyl (2 of 3), *p*-hydroxy (1 of 2) and *p*-acetamido (2 of 4) analogues.

Discussion. Although benzaldehyde thiosemicarbazone inhibits growth of the tuberculosis bacillus *in vitro*(1,7), it is relatively inactive *in vivo*(8,9). Derivatives of benzaldehyde thiosemicarbazone with ethylsulfonyl, acetamido(2), amino and dimethylamino(8,9) substituents in the *para* position of the benzene nucleus are very effective for the treatment of tuberculosis in experimental animals. These substances, however, have been observed to produce little or no protection against vaccinia infection in mice. In contrast, the unsubstituted compound exhibits a marked degree of protection of mice against vaccinia virus.

The mode of action of the thiosemicarbazones in tuberculosis has not been established. Domagk(10) has expressed the view that the therapeutic effects observed are the result of a direct action of these compounds on the tuberculosis organism and not a consequence of some theoretical indirect action on the host. In the case of vaccinia virus, it was observed that the compounds (No. 1, 8) which were most virustatic *in vitro* were the most effective materials in protection tests. Since the thiosemicarbazones do not appear to inactivate the virus as the result of prolonged contact, it may be assumed that they act, not by a direct virucidal action, but by interference with proliferation of the infectious agent. The inhibition of viral multiplication may be the result either of a direct action by the thiosemicarbazones on the virus or indirectly, possibly by alteration of the metabolic processes of the tissues of the host.

It is of interest to note that certain phenoxythiouracils protect mice against vaccinia virus(6). These compounds, like the thiosemicarbazones, contain a thiourea group. When administered in the diet, the dosage of phenoxythiouracils required to protect mice against vaccinia virus is 10 to 20 times greater than that of thiosemicarbazones. Active members of both groups of compounds are relatively insoluble in distilled water.

Summary. Benzaldehyde thiosemicarbazone prevents multiplication of vaccinia virus in chick embryonic tissues when present in a concentration of one μg per ml of medium. Substitutions in the *para* position of the benzene nucleus or in the 4-position of the thiosemicarbazone portion of the molecule reduce virostatic activity. 2. When fed in the diet, benzaldehyde thiosemicarbazone produces a marked degree of protection of mice inoculated intracerebrally with vaccinia virus. Derivatives with substitutions in the *para* position of the benzene nucleus or in the 4-position of the thiosemicarbazone portion of the molecule produce little or no protection against vaccinia infection in mice. 3. The thiosemicarbazones do not appear to inactivate vaccinia virus as the result of prolonged contact *in vitro*.

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Experimental Hepatic Coma. (18958)

A. M. Rappaport and Wallace N. Lotto. (Introduced by Charles H. Best.)

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While experimenting with ischemia of the liver in dogs, we observed 4 animals in comatose states which closely resembled hepatic coma in man. Two of these were fatal, 2 non-fatal. After many attempts to induce these comatose states at will, we found that a graded production of liver ischemia might result in the clinical manifestations of coma. If the increasing ischemia of the organ can be coped with by the gradually developing blood flow from collateral circulation, the animal may recover from coma.

Ischemia of the liver can be induced by gradually tying the vessels in the hepatic-duodenal ligament and diverting the blood flow from the portal vein into the vena cava (classical Eck fistula). The collateral circulation will then develop from the reversed flow in the gastro-duodenal, left gastric and phrenico-abdominal arteries(1).

Six 9 to 15 kg dogs were operated upon under nembutal anesthesia (35 mg/kg body weight) in a 2-stage procedure: 1st stage: Portal-caval anastomosis and ligation of the portal vein in the hepato-duodenal ligament (Eck fistula). 2nd stage: 24 to 48 hours later under nembutal anesthesia (only half the previous dose is now necessary) ligation of the common hepatic artery. Intensive treatment with penicillin and streptomycin was started immediately after the first stage and continued for about 7 days post-operatively. Four of the dogs were infused continuously with saline-glucose solution in order to avoid the complicating factor of hypoglycemia. Of the 2 dogs which did not receive glucose, one died in hypoglycemic convulsions. The other dog was in a subcomatose state for 36 hours and recovered. Of the 4 dogs infused with glucose, three had a non-fatal liver coma.

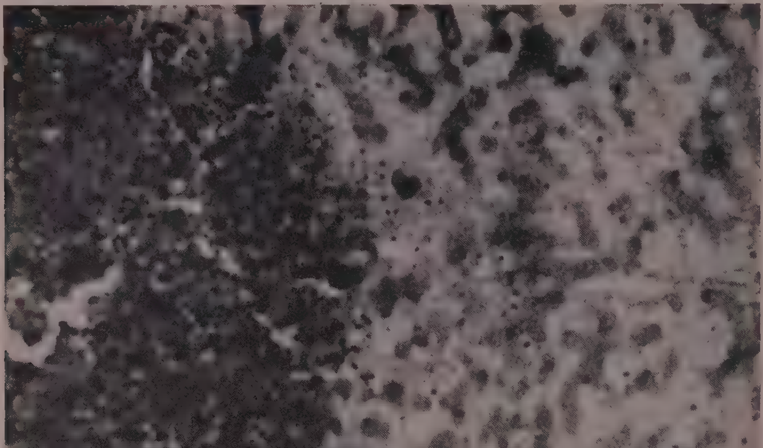

FIG. 1. Dog A64, 33 hr of fatal liver coma. (Azo-carmine aniline blue. × 300). Haemorrhagic necrosis around central vein.

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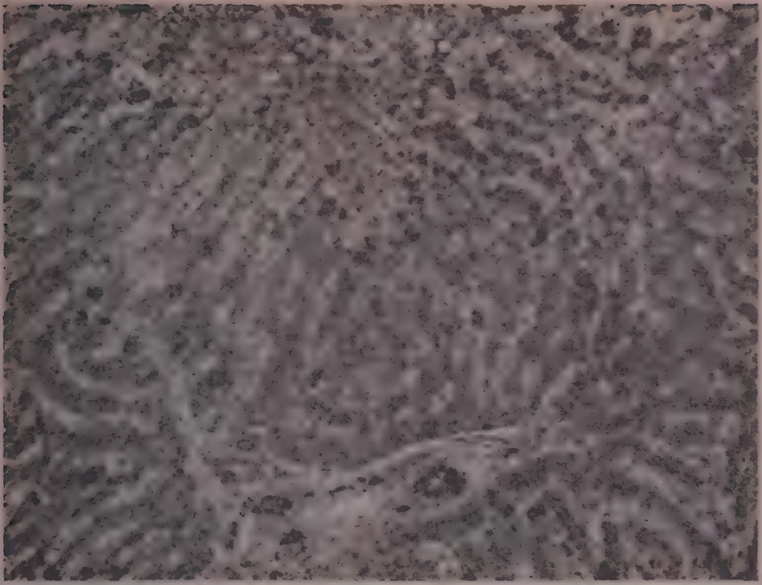

FIG. 2. Dog A69, 3 days of non-fatal liver coma. (Frozen section Oro & H × 200). Centrolobular necrosis with slight fatty change. The parenchyma around portal area is preserved and shows little fatty degeneration.

One dog died 33 hours after the second stage operation in a deeply comatose state.

The signs displayed by the dogs were: Restlessness, loss of appetite, vomiting, sometimes tarry stools, fever, jaundice, bradycardia, stupor, ataxia, somnolence, coma with deep sonorous breathing, muscular rigidity, convulsions, and muscular twitchings (soubresauts of the French literature). Pronounced fetor hepaticus was observed in some of the dogs with advanced ischemia of the liver. However, this classical sign was not prominent in the acutely comatose animals; but this situation requires much further study.

Chemical studies have been made in the Department of Pathological Chemistry, on the blood of animals in coma and after recovery. This work will be extended and the findings will be reported.

Two of the surviving dogs showed signs of a spinal cord lesion (spastic paraplegia) 2½ to 4 weeks after they came out of coma.

The histopathological report on the liver of the dog that died in coma made by Dr. Stanley Hartroft states: "Wide central hemorrhagic necrosis occupying the entire lobule. Here and there small haloes of non-necrotic periportal parenchymal tissue are present (Fig. 1)". One dog sacrificed 3 days after it recovered from liver coma showed evidence of centro-lobular necrosis throughout. In these regions fatty change was also apparent (Fig. 2).

From our preliminary findings, we may conclude: (1) Fatal or non-fatal hepatic coma can be produced in dogs by a gradually developed ischemia of the liver. (2) This ischemia can be induced by a two-stage operative procedure: (a) Eck fistula. (b) 24 to 48 hours later, ligation of the common hepatic artery. The shorter the time interval between the two operations, the more marked the signs of liver coma. (3) Intensive postoperative treatment of the dogs with antibiotics is necessary and glucose infusion is essential. (4) Some of the dogs surviving coma show signs of a spinal cord lesion.

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Distribution of Radioactive Phosphorus in Normal and Diseased Brain Tissue. Experimental and Clinical Observations. (18959)

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The purpose of this study is to determine the relative amounts of radioactive phosphorus in normal and diseased mammalian brain tissue following parenteral administration of the isotope. The clinical application of this isotope to the practical problems of brain tumor localization and delineation by other workers(1,2) stimulated interest in revealing what pathological change in tissue would alter the normal isotope content.

Methods. A. Experimental. Adult cats were utilized. A series of traumatic, purulent and ischemic lesions were made in the cerebral hemispheres of the animals. In addition, it was possible to make observations on the fetal brain tissue and the adult brain tissue of three pregnant animals. The animals which underwent surgical procedures received the radioactive isotope 5-6 days following operation in the cases of the traumatic and ischemic lesions and at varying intervals in excess of 6 days in the cases of the purulent lesions. The isotope was administered by vein in the form of weak phosphoric acid in doses varying from 9 to 39 microcuries of P-32 per kilo body weight, the average dose being 18 microcuries per kilo. The animals were sacrificed in from 24 to 96 hours after the injection, and the brain tissues were removed and sectioned. The areas of lesions were removed en bloc and were matched by control blocks of tissue from the contralateral hemisphere. Cerebellar and brain stem blocks were sampled in certain instances also. The tissues were chilled, weighed in the wet state and homogenized with distilled water to a consistency which permitted their being drawn into a 1.0 ml serological pipette. 1.0 ml aliquots of the homogenates were placed in dry, weighed metal capsules for drying and assay. Similar aliquots were fractionated into the acid soluble, phospholipid and nucleic acid containing constituents by the method of Schneider(3,4). These chemical fractions were dried in capsules for assay. The dried films were assayed for radioactive content in lead-shielded counting chambers by the use of a Geiger-Muller, bell type counter (2.0 mg/cm^2 window thickness, Background 0.45 counts/second). A minimum of 3500 counts was taken, and in all instances where a sufficient material was available duplicate determinations were made. B. Clinical. Brain tumor cases anticipating surgery received sterilized solutions of the weak phosphoric acid containing the isotope in doses approximating 7 microcuries per kilo body weight. The material was given by vein 12 to 48 hours before operation. Control tissue was biopsied at operation to compare with the abnormal tissue. The tissues were treated in the same way as outlined above.

Results. The results of the tissue assay were expressed (a) in counts per second per gram dry weight of tissue and (b) in a ratio, termed the "differential absorption ratio" (D.A.R.) which is obtained by dividing the actual counts per second of one gram of specimen (calculated as wet weight) by the theoretical count of an equal weight of tissue if the isotope were uniformly distributed throughout the body. Such an expression, while it has no absolute value, is useful in comparing the results from different animals and cases in which varying the dose of isotope per unit body weight could not be avoided. The results of assay of the tissue fractions were expressed in percent of recovery of the total isotope content.

A. *Experimental Material.* 1. *Fetal tissue.* (3 animals). 24 hours after the administration of the isotope the fetal brains showed small differences in assay from the maternal tissue of the same animal. Forty-five and 48 hours after injection the dry weight values of the fetal brains rose to 2.5-4.0 times the maternal brain values. The differential absorption ratios (D.A.R.'s) revealed definite but less striking differences in favor of the fetal tissue. Approximately 50% of the isotope was recovered in the acid soluble fractions of the maternal tissues while 60% was present in this fraction in the assay of the fetal tissues.

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	Ratio of C/S/G dry wt		D.A.R.	
	Adult	Fetal	Adult	Fetal
24 hr	1	1.9	.12	.12
45 hr	1	2.5	.26	.35
48 hr	1	4	.19	.45

2. *Traumatic lesions.* (6 animals). Higher isotope assays were observed in the traumatized areas of the hemispheres than in control hemisphere tissue of the same animals. Lesions produced by the electrosurgical unit showed dry weight assays 3.6 and 3.7 times higher than control tissue assays. Lesions produced by blunt instruments showed smaller differences. In all instances more isotope was recovered in the acid soluble tissue fraction than in any other.

	Ratio of C/S/G dry wt		D.A.R.	
	Control	Lesion	Control	Lesion
Blunt instrument	1	1.2	.15	.25
	1	1.7	.21	.38
	1	1.9	.24	.62
	1	2.9	.07	.18
Electro-unit	1	3.6	.13	.50
	1	3.7	.20	.80

% recovery in chemical fractions (avg figures)

	Control	Lesion
Acid soluble	57	49
Phospholipid	25	27
Nucleic acid	Not significant	

3. *Purulent lesions.* (7 animals). No walled off abscess was produced. In 2 animals grossly localized collections of liquefied pus were produced. The dry weight assays of the tissue of the lesion gave values from 3-3.5 times the control assays. One animal with an area of localized meningitis and a ventriculitis but without an area of cerebral suppuration showed an assay in the altered area of 1.7 times the control. The other 3 animals, one with inspissated pus and agar in its ventricle and 2 with only minimal cortical contusions revealed only minor deviations from the normal values of the control tissues.

	Ratio of C/S/G dry wt		D.A.R.	
	Control	Lesion	Control	Lesion
	1	.9	.16	.17
	1	1.2	.12	.14
	1	1.4	.19	.30
	1	1.7	.10	.19
	1	2.9	.27	.66
	1	3.6	.14	.54
Foreign body	1	9.1	.09	.55

A single experiment in which "Saraka" seeds were implanted into the cerebral hemisphere revealed higher assays in the liquefied mass and surrounding "edematous" brain tissue than in the case of any purulent lesions studied.

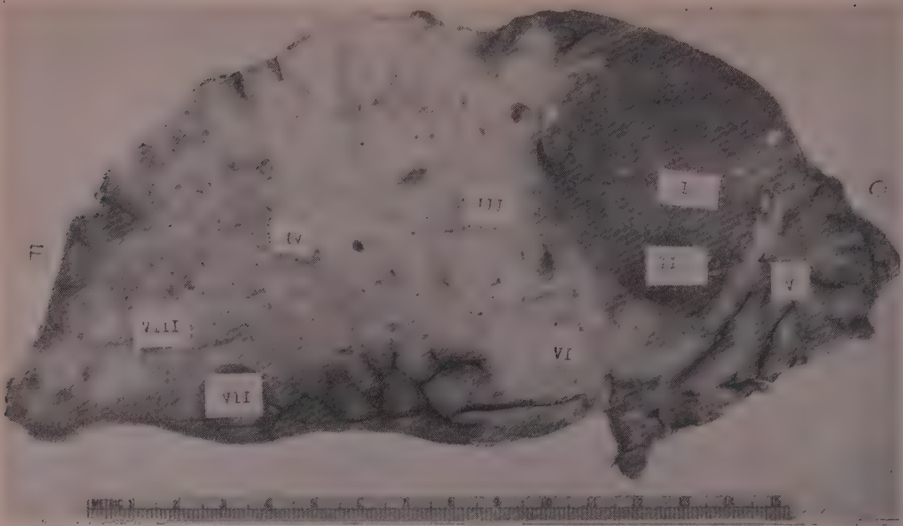
4. *Ischemic lesions.* (4 animals). Hemorrhagic softenings were produced by clipping and coagulation of the middle cerebral complex of vessels in the Sylvian fissure. The central portions of the lesions as well as the adjacent cerebral tissue demonstrated dry weight assays of 4.0-4.5 times the values of the opposite hemisphere. The D.A.R.'s showed slightly smaller differences. The relative isotope concentration in the chemical fractions did not appear different between the abnormal and control tissue. Two animals which developed no ischemic lesions showed no important differences between the tissues assayed excepting where a local area of coagulation necrosis raised the isotope content.

	Ratio of C/S/G dry wt		D.A.R.	
	Control	Lesion	Control	Lesion
No infarct	1	.9	.07	.06
	1	1.5	.07	.12
Gross infarcts	1	4.3	.12	.41
	1	4.8	.12	.50

Assay of the chemical fractions revealed no consistent alteration from normal values in the lesions produced.

% recovery in chemical fractions (avg figures)

	Control	Lesion
Acid soluble	52	49
Phospholipid	26	27
Nucleic acid	Not significant	



Tissue location	I	II	III	IV	V	VI	VII	VIII
Counts/sec/gram dry wt	96.61 103.06	828.15 811.40	72.30 79.47	2.87 3.38	9.77 10.18	22.37 23.61	12.25 13.04	6.07 —
Differential absorption ratio	0.052	0.488	0.050	0.003	0.009	0.016	0.009	0.006

Glioblastoma Multiforme 19 hr after 6.6 μc $_{15}P^{32}$/kg

FIG. 1. Analysis of P^{32} content in brain slice. Note correspondence between area I within the tumor's necrotic area and area III in uninvolved white matter.

B. *Clinical material.* Tissues from 8 brain tumor cases were obtained as surgical specimens. In every case the tumor tissue assay exceeded the control tissue assay several-fold. The ratios of the dry weight content of isotope of control vs. tumor tissue were as follows:

Tumor type	Control		Tumor
Glioblastoma multiforma	1	:	7.7
	1	:	12.8
	1	:	18.6
	1	:	64.8
Ependymoblastoma	1	:	22.6
Astrocytoma	1	:	6.2
Chromophobe adenoma of pituitary	1	:	3.6
Unclassified (glioma?)	1	:	7.5

Sufficiently large specimens in 2 of the glioblastoma cases permitted the assay of soft, necrotic tissue within the tumor mass as well as firm, neoplastic tissue. The isotope content of the degenerated tissue was several times below that of the firm tissue. The ratios of the dry weight assays were as follows:

Control		Necrotic area		Firm area
1	:	3.7	:	18.6
1	:	7.9	:	64.8

One of these 2 cases is illustrated (Fig. 1) in a manner to show the areas of specimens which were sampled and assayed. Histological study of the same areas provided evidence that there was no tumor tissue in areas III to VIII. Area I was soft and necrotic whereas area II was firm, cellular tumor. The assay figures are tabulated beneath the brain slice. Assay of the chemical fractions revealed the major portion of the recovered isotope to be in the acid soluble fractions of both tumor and control tissues. There was a tendency for the relative content of isotope to be higher in the phospholipid and lower in the acid soluble fractions for the tumor tissue than for the control tissue. These variations did not exceed 10% of the amount of recovered isotope.

% recovery in chemical fractions (avg figures)

	Control	Tumor
Acid soluble	74	69
Phospholipid	9	15
Nucleic acid	Not significant	

Discussion. It appears from these observations that the lesions of the brain which were studied have a higher content of P-32 following its intravenous administration than the corresponding control brain tissue of each preparation. We cannot reason from the results of animal work to those of clinical material, but it is noteworthy that in general the 8 brain tumor cases showed higher ratios between control and abnormal tissue than did the experimental lesions. Areas of the brain tumors which were degenerating or necrotic revealed lower values than firm areas of tumor. It would seem that areas of brain tissue destruction, whether in experimental lesions or in areas of tumor degeneration, exhibit higher isotope content than normal brain but usually lower values than "viable" brain tumor tissue.

Several factors are thought to influence the uptake of radioactive phosphorus by tissues in general(5-7). (a) The rate of phosphorus turnover or exchange in a given tissue(8,9). (b) The building of new tissue of which phosphorus is an essential constituent(8,9). (c) Mitotic activity(10,11). (d) Temperature(12). (e) Membrane permeability (12,13). (f) The total amount of exchangeable phosphorus in the tissue(9). It is probable that not all of these factors are of importance in this study. Although the total content of phosphorus in normal brain tissue is low in comparison with other soft tissues, the isotope uptake is low and is accomplished at a slow rate(9,14). Equilibrium is reached at a lower level and at a slower rate than in the case of most all other soft tissues of experimental animals studied(15-19). The differences in distribution of the isotope in the chemical fractions between the experimental lesions and control tissues were not of sufficient magnitude to explain the total tissue differences on the basis of any disproportionate increase of the isotope in a given fraction. The destructive processes which broke down capillary and other tissue membranes may have led to changes in permeability to phosphate ions both at the capillary and cell membrane barriers. Such a mechanism is an attractive explanation for the differences observed. However, in the absence of studies comparing the isotope content of the plasma, extracellular and intracellular spaces, we are not justified in passing judgment on the probable importance of such a mechanism.

Observations upon the distribution of phosphorus in brain tumors are few. Cumings(20) reported the vast bulk of the stable element to be in the acid soluble fraction with very little nucleoprotein phosphorus and less phospholipid phosphorus. Erickson and associates (21,22), employing Schneider's methods, found the phosphorus distributed far more evenly between the fractions. However, as has been well shown by Fries and Chaikoff (18) the stable phosphorus distribution may have no relationship to the percentage of total

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radioactive isotope which can be recovered in any given fraction. Our findings agree with those of Erickson and associates(22) who found the P-32 in decreasing amounts in the acid soluble, phospholipid and nucleic acid fractions. Unlike the experience of others with other neoplastic tissue(23,24) we did not find an important alteration in the percentage distribution of the isotope in the chemical compartments compared with control tissue. We recognized that our observations may not have covered a sufficiently long period following the administration of the isotope. From our data, however, we are unable to explain the increase in total tissue content by, for example, a sharp rise in isotope-containing nucleic acid. By studies of the nuclear content of P-32 Marshak(10,11) was able to reach conclusions suggesting the importance of mitotic activity in determining the increases of the isotope above normal in tumor and regenerating tissue of livers of experimental animals. We are unable to provide any data on this point. We would suggest that there are factors at play which influence the brain tumor increases of isotope which are not functioning in the case of the experimental lesions we have studied.

Conclusions. 1. Traumatic, purulent and ischemic lesions of the brains of cats demonstrate increased contents of radioactive phosphorus following intravenous administration of the isotope when compared with control brain tissue. 2. Fetal cat brain tissues demonstrate higher contents of the isotope in 45 and 48 hours after administration than maternal brain tissue of the same animal. 3. Brain tumor tissue from clinical cases reveals greater deviations above the normal of any pathological nervous tissue studied. When the tissue architecture of the tumor is intact the content of the isotope is higher than in degenerating, softened tissue of the same tumor mass. 4. The distribution of the isotope among the three phosphorus containing fractions of the pathological tissues does not show sufficiently marked alterations from the distribution in control tissues to explain the abnormal increases in total tissue content on the basis of a disproportionate increase of any one isotope-bearing fraction. It is suggested that different mechanisms are at play to explain the increase of isotope in destroyed tissue of the brain as compared with the mechanisms at play in neoplastic tissue. 5. Caution must be employed when utilizing radioactive phosphorus in brain tissue to localize or delineate tumor masses by probe methods, for non-tumor tissue may show a differential ratio between normal and abnormal tissue such as to cause errors in interpretation.

The authors are pleased to acknowledge the helpful advice of Dr. A. Earl Walker in whose department this work was carried out. Clinical material was provided through courtesy of Dr. Walker, Dr. Herbert C. Johnson and Dr. Robert G. Fisher. The National Research Council defrayed part of the expense of this study, and the Atomic Energy Commission furnished the P-32. Dr. J. Logan Irvin of the Division of Physiological Chemistry was generous in his suggestions aiding the biochemical procedures.

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Some Effects of Alloxan on the Canine Kidney, with Special Reference to the Glomerulus.* (18960)

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During a long term study of experimental diabetes mellitus in dogs, 2 animals were encountered which failed to become diabetic after repeated injections of alloxan. In these, the development of renal lesions was followed by serial biopsies. In addition to the tubular damage previously described by others(1-6), we have observed the late appearance of glomerular degeneration, a finding hitherto unreported in the dog.

Method. Young mongrel dogs aged 4-6 months were given alloxan monohydrate (Eastman Kodak Co. or Schuylkill) intravenously in amounts ranging from 50-100 mg per kilo of body weight. Biopsies of the kidney were performed at intervals of from one to several months afterward. All animals were kept in metabolism cages and maintained on constant diets containing ground horse meat, bread, brewer's yeast, and cod liver oil.

Results. Pathological observations. 1. Dog 132. This animal received a total of 3672 mg of alloxan in 4 doses over a period of 5 months (70 mg/kg on 11/19/47 at age 4 months and on 3/11/48; 80 mg/kg on 3/24/48; 100 mg/kg on 4/7/48).

One and one-half months after initial dose of alloxan. The kidney appears normal except for an occasional tiny, pale blue hyaline cast and rare scattered atrophic tubules.

Five and one-half months after initial alloxan. (Fig. 1). The kidney shows moderately severe changes, consisting of some interstitial edema and fibrosis, considerable atrophy of tubules, a few scattered tubules filled with amorphous debris and without tubular epithelium, and a few dilated tubules. The glomeruli are comparatively normal ex-

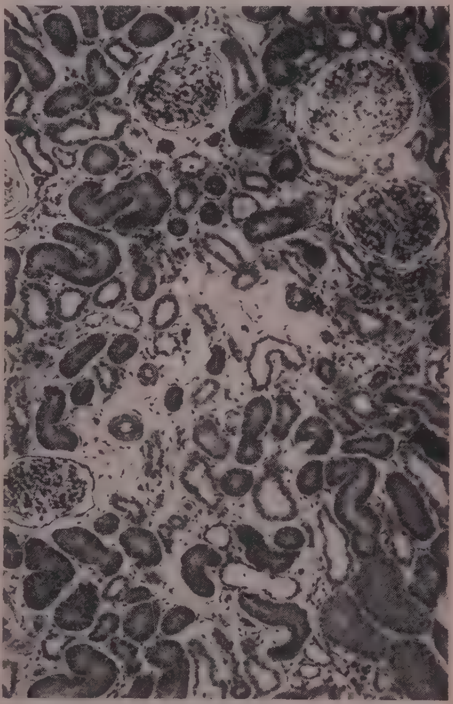

FIG. 1. Photomicrograph (× 110). (Dog 132, 4-29-48). Renal biopsy (Hematoxylin and eosin) taken 22 days after the last of a series of 4 injections of alloxan totalling 3672 mg over a period of 5 months. There is much atrophy of tubules but minimal glomerular damage at this stage. Three relatively normal tubules are seen in the lower right corner.

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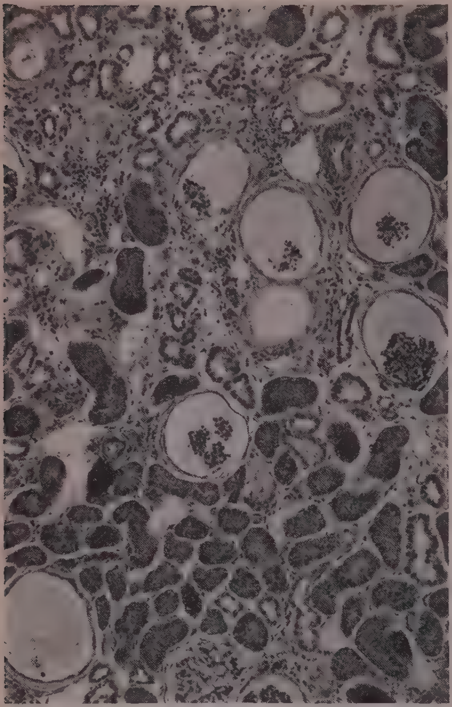

FIG. 2. Photomicrograph (× 110). (Dog 132, 2-3-49). Renal biopsy taken about 9 months later. In addition to extensive tubular atrophy and interstitial fibrosis, glomerular changes are now conspicuous. They consist chiefly of atrophy of some tufts, and dilation of some Bowman's spaces but shrinkage of others.

cept that a few are atrophic and lie in dilated spaces. The arterioles and small arteries are conspicuous but probably not thickened.

Fourteen and one-half months after initial alloxan. (Fig. 2). The tubular changes are more striking than at 5½ months and glomerular changes are now also evident. While some tubules appear comparatively normal, many show severe atrophy and a few are dilated. Interstitial fibrous scars are now more conspicuous due apparently in part to disappearance of some tubules. Such areas seem to have an increased number of glomeruli. About half of the glomerules are relatively normal. The remainder show great atrophy of glomerular tufts; a few are fibrotic. Bowman's spaces are usually dilated, sometimes greatly, but a few are shrunken; there is much fibrous tissue increase around most of the abnormal glomeruli, but their epithelium is thin and flat. They exhibit neither crescents nor synechiae. There is no fatty change and the vessels are not unusual.

Thirty months after initial alloxan. The degenerative changes appear less severe than at 14½ months, possibly because of either a difference in sampling or some degree of recovery. Most of the tubules appear relatively normal as do the majority of the glomeruli. The remainder show atrophy of the glomerular tuft, without epithelial changes, and most of such glomeruli are now shrunken. A few glomerular tufts are fibrosed. A few arteries have thick but probably not thickened walls. There is no fatty change or glycogen deposition.

2. Dog 199. This animal received a total of 2151 mg of alloxan in 3 doses over a period of 6 weeks (75 mg/kg on 6/3/48 at age of 4 months; 60 mg/kg on 7/10/48; 85 mg/kg on 7/20/48).

Two months after initial dose of alloxan. (Fig. 3). This kidney shows severe degenerative change which is chiefly tubular but also glomerular. While a few tubules appear nearly normal, the majority show severe atrophy and many have disappeared. There is, therefore, a relative and probably an absolute increase in interstitial fibrous tissue, together with some edema and an apparent increase in glomeruli. Leukocyte infiltration is minimal. Some atrophic tubules contain small casts and Bowman's spaces show a pinkish precipitate. A few of the atrophic tubular cells show stainable lipid. Practically no normal glomeruli are seen. Most of them exhibit atrophic tufts, some of which lie in dilated and others in contracted Bowman's spaces. Many of the abnormal glomeruli show a marked increase of fibrous tissue in Bowman's capsule but there is no epithelial proliferation. The blood vessels appear normal.

Eight months after initial alloxan. The renal changes are of the same variety as at 2 months, but while they are less extensive they are nevertheless more advanced, possibly owing to a difference in sampling, or in repair, or both. About half of the glomeruli and

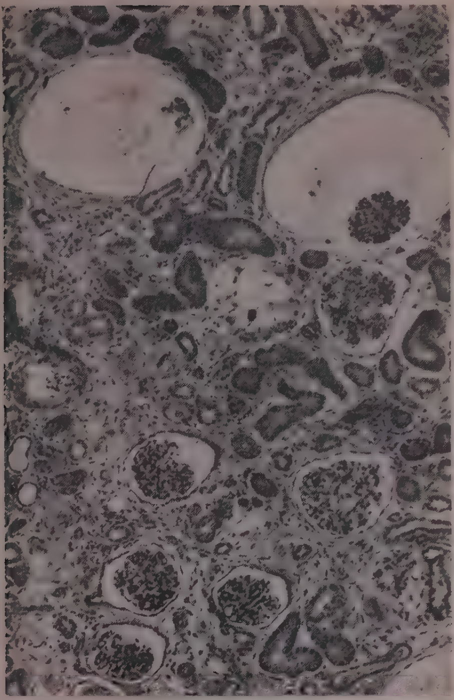

FIG. 3. Photomicrograph (× 110). (Dog 199, 8-12-48). Renal biopsy taken 23 days after the last of a series of 3 injections of alloxan totalling 2151 mg over an interval of 6 weeks. Tubular and glomerular changes are similar to but more severe than those in Dog 132.

many tubules appear relatively normal. Other tubules are atrophic or have disappeared, leaving fibrous scars. A few show a small amount of stainable lipid. Most of the abnormal glomeruli are greatly dilated and enclosed by dense fibrous tissue. They contain tiny atrophic glomerular tufts and a pink-staining deposit. The epithelium is thin and flat, and there are no crescents or synechiae. A few glomeruli are shrunken and in some areas they appear abnormally numerous. A few afferent arterioles appear thickened.

Miscellaneous observations. Proteinuria, despite severe renal damage, was only occasional, transitory and usually minimal. The blood urea nitrogen or nonprotein nitrogen rose moderately soon after alloxan administration but declined quickly to normal values. In dog 132 a reducing substance appeared in the urine after 34 months, the glucose tolerance curve being normal. This animal subsequently became frankly diabetic.

Discussion. Recently Mann and Goddard (7,8) have found that male rats made diabetic with alloxan monohydrate developed well-marked progressive lesions in the renal glomeruli as early as 50-90 days. Primarily affected were the walls of the capillary tufts and the parietal layer of Bowman's capsule. Pyelonephritis was a frequently associated condition. Tubular changes were not described. Renal arteriolar lesions were absent. The incidence of glomerular damage could not be correlated with the duration or severity of diabetes, or with the nature of the diet. No lesions were found in non-diabetic, alloxan-injected controls.

Obviously unrelated to alloxan, and possessing their own special significance, are renal changes found in experimental diabetes produced by other means. Thus, Lukens and Dohan(9) described in the kidneys of a dog made permanently diabetic with anterior pituitary extract changes which appeared to be the counterpart of the intercapillary glomerulosclerosis of human diabetes. The development of glomerular degeneration in depancreatized rats has been reported by Foglia and associates(10). The lesions did not appear until more than two months after 95% pancreatectomy. The earliest change was hyaline thickening of the glomerular arteriole and capillaries, and finally sclerosis of the tuft. These lesions were diffuse and severe, but no hyaline masses similar to those described by Kimmelstiel and Wilson(11) were seen.

The observations here reported are, so far as we are aware, the first in which definite glomerular lesions in the dog following administration of alloxan have been described. It is noteworthy that they occurred after mul-

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tiple doses of the drug and that injury of this degree was never seen in many other dogs, including 2 littermates of dog 132, which survived a single dose. Serial biopsies showed, in agreement with earlier reports and with our own observations on other dogs, that the primary effect of alloxan was on the tubules. The glomerular changes lagged behind the tubular degeneration and they appeared both in time and in type to be consequent to the latter. The histologic picture suggests that the glomerular damage originated from a failure of drainage, with resulting dilatation of Bowman's space and atrophy of the tuft. The absence of epithelial and endothelial proliferation and the presence of periglomerular fibrosis are consistent with this interpretation.

Summary. In 2 dogs which did not become diabetic following repeated doses of alloxan, serial biopsies of the kidney showed the late development of glomerular lesions. These lagged behind the usual tubular degeneration and appeared both in time and in type to be consequent to the latter. Similar studies on dogs receiving a single dose of alloxan and surviving for comparable periods of time showed only minimal glomerular changes.

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V. Effect of Epinephrine, ACTH and Cortisone on Citrate, Calcium, Glucose and Phosphate Levels in Rabbits.* (18961)

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The effect of administration of glucose, insulin and epinephrine on serum citrate levels in humans has been studied(1-3). Administration of both glucose and insulin resulted in a fall in citrate levels, and administration of epinephrine resulted in a rise in citrate levels for several hours. That administration of glucose will result in a drop in citrate levels has been confirmed(4,5). Evidence has been presented to suggest that epinephrine stimulates secretion of ACTH and in turn the corticoid hormones(6). For this reason it was decided to investigate the changes in citrate levels occurring in blood under the influence of these hormones.

Serum calcium(7) and inorganic phosphate (8) estimations were carried out in addition to citrate(9,10) and glucose(11). In the case of epinephrine potassium levels(12) were also studied. These additional studies were undertaken because a relationship exists between serum calcium and citrate levels and ionic calcium, citrate and phosphate levels due to the formation of poorly ionized calcium complexes(13). Female rabbits weighing be-

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* The fifth study on mechanism controlling serum citrate levels. Supported in part by a grant from the Dazian Foundation.

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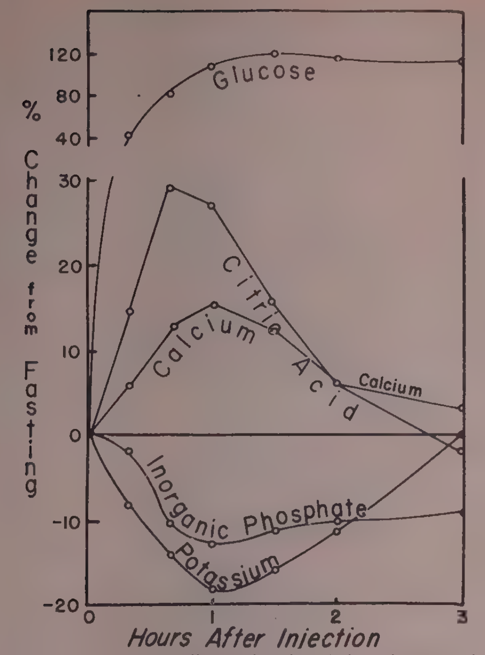

FIG. 1. Average effect of epinephrine (0.25 mg) on serum glucose, citric acid, calcium, inorganic phosphate and potassium.

tween 2-4 kilos were fasted for at least 48 hours to ensure stable initial levels of the various constituents of blood to be studied. Blood citrate levels are affected by food intake and this species is well known for long retention of food in its intestinal tract. The procedure consisted of drawing fasting blood from the ear. The hormone was immediately injected. Blood samples were taken at intervals (Fig. 1-3).

Fig. 1 summarizes the results obtained with epinephrine administered subcutaneously (0.08 mg/kilo). The curves illustrate the average results obtained with 26 rabbits for all values except potassium levels which are averaged for 11 rabbits. The values are the average change from the fasting. The curves for the individual rabbits resembled closely the shape of the average curves.

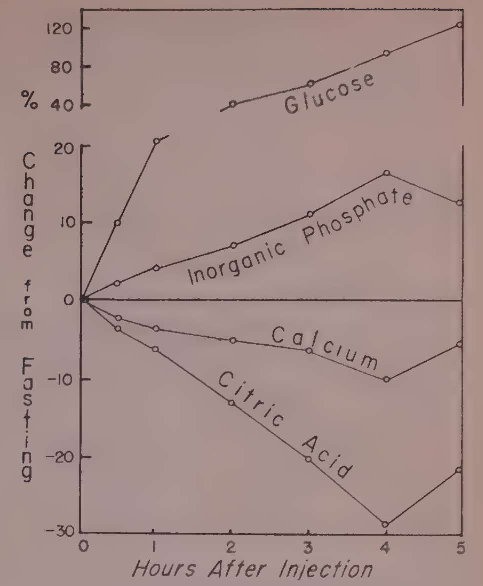

FIG. 2. Avg effect of cortisone (25 mg/kilo of body wt) on serum glucose, inorganic phosphate, calcium and citric acid in 10 rabbits.

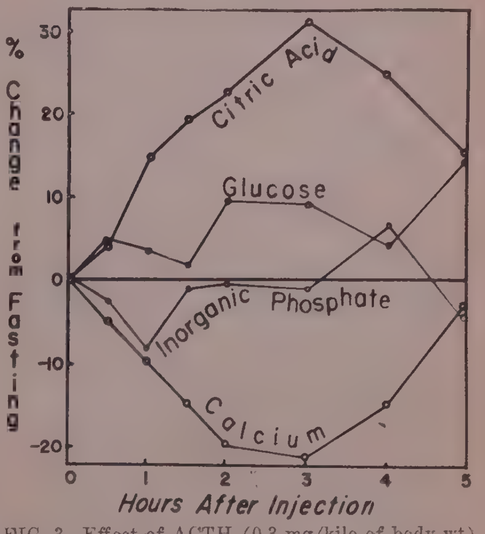

FIG. 3. Effect of ACTH (0.3 mg/kilo of body wt) on serum citric acid, glucose, inorganic phosphate and calcium.

Fig. 2 shows the results obtained with cortisone injected as a saline suspension intramuscularly. Ten animals were used in these experiments. The response of the individual rabbits was similar to the average curve, maxima and minima occurring at approximately the same times.

Fig. 3 illustrates the results obtained with

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TABLE I. Effect of Intramuscular Administration of ACTH (0.6 mg/kilo) on Calcium and Phosphorus Serum Levels in Rabbits After Fasting for 48 Hr.

Rabbit #	mg/100 ml	Fasting	½ hr	1 hr	1½ hr	2 hr	3 hr	4 hr	5 hr
88	P.	3.28	3.28	3.39	3.54	5.04*	—	4.68	3.75
	Ca.	13.6	12.6	12.2	11.9	11.2	—	10.7	10
89	P.	4.07	3.39	3.13	3.19	3.39	3.57	3.60	3.66
	Ca.	12.6	11.9	11.6	11.3	10.2	12	12.4	12.7
90	P.	4.28	4.07	3.68	3.45	3.45	3.54	3.57	3.90
	Ca.	12.1	11.6	9.9	9.1	8.9	10.2	11.8	11.2
91	P.	2.97	3.25	3.36	—	3.39	2.86	2.36	2.26
	Ca.	12.8	12.9	12.8	—	12.5	11.7	10.9	12.6

* Convulsions.

TABLE II. Effect of ''Massive'' Doses of ACTH on Serum Citrate, Calcium, Phosphate, and Glucose Levels on Fasting (48 Hr) Rabbits.

Rabbit No.	Dose, mg/kilo	Maximum % change from fasting*				Time of death, hr
		Citrate	Calcium	Phosphate	Glucose	
32	.75	+63	—31	——	+82	3
54	1	+43	——	—13	+83	survived
59	1.5	+50	—40	+57	+45	10
33	1.5	+57	—26	——	+110	2
58	1.5	+50	—40	+57	+45	10
67	2	+105	— 2	+240	+10	1½
66	2	+77	—29	+58	+75	survived
68	2	+55	—17	—28	+6	,,
69	4	+125	—35	—15	+90	,,
70	5	+26	—51	+17	+46	,,
71	5	+44	—25	+6	+130	,,
72	6.5	+50	— 3	+156	+82	1

* Mean fasting values found in this study (mg/100 ml serum), citric acid 3.67 (66 rabbits), glucose 120 (56 rabbits), calcium 13.8 (42 rabbits), inorganic phosphorus 4.86 (43 rabbits).

ACTH injected intramuscularly in 12 rabbits. The material used was taken from two samples (Armour Standard Clinical La-1A and Armour Animal Lot No. 128-105), dissolved in saline to contain 1 mg/ml and administered in doses of 0.3 mg/kilo. Changes in citrate and calcium levels were consistent and are shown in Fig. 3. Glucose and phosphate changes were somewhat variable. However, the average curve in Fig. 3 for these constituents indicates the general tendency.

Table I lists calcium and phosphorus results for 4 animals on a dosage of 0.6 mg/kilo.

Table II summarizes the results obtained with larger doses of ACTH.

Discussion. Both cortisone and epinephrine (Fig. 1, 2) cause a rapid rise in glucose levels. With respect to calcium, citrate and phosphate levels the response of the two hormones was in opposite directions. The rise in citrate levels on epinephrine administration was similar to that observed in humans(3). Changes in total calcium values were counterbalanced in each case by a change in citrate levels in the same direction. Thus the fall in calcium concentration when cortisone is administered is modified in its effect by a drop in citrate concentration which is known to complex calcium ions. Similarly, the rise in total calcium with epinephrine is also modified by a rise in citrate concentration. The inorganic phosphate moves in the direction of maintaining a constant calcium to phosphate product, the change in phosphate being inverse to the change in calcium in both cases. The animals treated with cortisone were not in distress in doses up to 50 mg/kilo, administered to a few animals.

With ACTH the drop in calcium level was more pronounced than that observed with cortisone and a significant increase in citrate levels took place. Thus calcium levels and citrate levels both moved in the direction which would lower calcium ion concentration. In this respect *the biochemical effects of cortisone and ACTH are quite different.*

The effect of ACTH on glucose levels was markedly different from that observed with cortisone in rabbits. With cortisone the glucose levels rose promptly and had not reached their peak at the end of 5 hours when the glucose level was twice the fasting level. With ACTH no such dramatic rise was observed (Fig. 2, 3).

The effect of ACTH in causing a reduction in calcium levels prompted us to continue this study on larger doses. In 4 cases only calcium and phosphate determinations were done. By limiting ourselves to these two constituents we hoped to check precisely the effect of ACTH on these levels. It was clearly demonstrated by this set of experiments, checked by triplicate analyses, recoveries and standards, that calcium levels were definitely lowered approximately 30% by a dosage of 0.6 mg/kilo of ACTH. All 4 animals were in distress, breathing rate increased, the animals dropped their heads and became incontinent at the end of one hour. Rabbit No. 88 convulsed at the end of 2 hours and responded with convulsions when approached at the end of 3 hours. This animal survived. It will be noted that in this animal, at the onset of convulsions, the phosphorus level rose to 40% above the fasting level. At the end of 4 and 5 hours this animal still showed an elevated phosphate level. The other 3 animals showed a phosphate minimum. A lowered calcium level accompanied by a lowered phosphate level is unusual in that it is well known that calcium and phosphate levels tend to change inversely so that their product remains constant. Under the influence of ACTH this relationship does not seem to hold. The sudden rise in phosphate level of the animal which had convulsed would only tend to further lower calcium ion concentration along with the rise in citrate which we had reason to believe has taken place at this point (Fig. 3, Table II).

Table II lists the results obtained with higher doses of ACTH. Convulsions and death were observed in 6 out of the 12 animals treated. The 6, who survived, were in severe distress. These animals tended to convulse when stimulated. Protein analysis, not shown in the table, demonstrated that no significant change in protein level had taken place. Thus the rise in citrate and phosphate probably resulted in a marked lowering of calcium ion concentration. A fall in protein level would have a compensating effect on a rise in citrate level as concerns the ionization of calcium.

The effect of the pituitary and adrenal hormones on citrate and calcium levels, demonstrated here, may have some bearing on normal and abnormal calcification and the problem of convulsions.

Summary. 1. Epinephrine subcutaneously causes a rise in calcium, citrate and glucose levels and a fall in phosphate and potassium levels. 2. Cortisone intramuscularly causes a rise in glucose and phosphate levels and a fall in calcium and citrate levels. 3. ACTH intramuscularly causes a rise in citrate and a fall in calcium levels. 4. It is suggested that the balance between ACTH, epinephrine, insulin and cortisone secretion, because of their effect on citrate, calcium and phosphate levels, play a significant role in the maintenance of the calcium ion concentration of the plasma.

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The Potentiating Effect of ACTH and of Cortisone on Pressor Response to Intravenous Infusion of L-Nor-Epinephrine.* (18962)

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Despite extensive investigation of the pharmacodynamic and therapeutic action of adrenocorticotrophin (ACTH) and cortisone, there have been but few reports on the effects of these agents on the circulation. Several reviews have summarized current knowledge (1,2). Adrenal steroids have been shown to prevent shock in adrenalectomized dogs(3) and to sustain experimental renal hypertension in adrenalectomized rats(4). Progressive insensitivity to nor-epinephrine has been noted in adrenalectomized dogs subjected to stress. Reactivity to nor-epinephrine was restored by adrenal cortical extract (ACE) but not by desoxycorticosterone acetate glucoside(5,6). Single injections of ACE potentiated the blood pressure response to previously ineffective concentrations of nor-epinephrine in adrenalectomized dogs but not in normal controls(7).

In humans treated with ACTH or cortisone hypertension has been irregularly noted (8-10). A rise in the tetraethylammonium chloride (TEAC) floor has been described in normotensive and hypertensive patients receiving ACTH and cortisone(11). However, others have noted(12) no definite change in the pressor responsiveness to epinephrine, nor-epinephrine, or tetraethylammonium chloride in patients treated with cortisone and only slightly increased pressure responses in patients treated with ACTH. In normotensive human subjects, the pressor effect of epinephrine and nor-epinephrine has been enhanced by pre-treatment with desoxycorticosterone acetate(13).

The present communication describes the effect of cortisone and ACTH on the pressor response of normotensive human subjects to 1-nor-epinephrine.

Methods. Three normotensive patients were studied. Repeated tests were performed in the same laboratory by the same personnel. All experiments in the same patient were carried out at the same time of day, from 1 to 3 hours after a light meal. An intravenous infusion of 5% glucose in water was administered through a No. 19 gauge needle into an antecubital vein. A constant rate infusion apparatus with variable gear ratios (Braun, Melsungen) was connected to the D/W infusion by a No. 25 needle inserted into the rubber tubing of the intravenous apparatus close to the needle in the antecubital vein. Two milliliters 1-nor-epinephrine, 1:1,000† was diluted in 250 ml of 5% dextrose in water. This could be administered at the rate of 0.26, 0.45, 0.72, 1.1 and 1.7 ml per

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* This investigation was aided by a grant from the William F. Milton Fund, Harvard Medical School, Boston, Mass.

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† Kindly supplied through the courtesy of the Winthrop-Stearns Research Institute.

minute. The heart rate was calculated from electrocardiograms (direct writing electrocardiograph); the blood pressure was measured by the auscultatory method. When all apparatus had been attached and the glucose infusion had been started, the pulse rate and blood pressure were measured every 2 minutes until a steady state had been achieved (approximately 30 to 40 minutes). The infusion of 1-nor-epinephrine was then begun and that of dextrose in water was stopped by clamping just distal to the insertion of the No. 25 needle. The observations were repeated every minute for 5 minutes and then every 2 minutes until a steady state could be demonstrated (usually at least 12 to 14 minutes). In 2 patients (Cases 1 and 2) the initial infusion speed of 0.45 ml per minute (3.6 µg per minute) was followed by similar observations at 3 more rapid rates of infusion of nor-epinephrine (5.8, 8.8 and 13.6 µg per minute). In the third patient (Case 3) the infusion of nor-epinephrine was maintained at 0.26 ml (2.1 µg) per minute, for 30 to 40 minutes.

Results. Case 1, M.B., a 28-year-old male, BIH No. M17695 entered for therapy of erythema nodosum and rheumatoid arthritis. During the first 9 days of hospitalization salicylates, tripelennamine hydrochloride (pyribenzamine) and penicillin were prescribed; there was no change in the clinical course. On the 10th day cortisone administration was started, 100 mg twice daily for five days and then 50 mg twice daily. Dramatic subsidence of joint and skin pain and fever were noted shortly after administration of cortisone(14). Fig. 1 demonstrates the results of infusions of nor-epinephrine in this patient before cortisone and 24 hours after starting oral cortisone. During the period of cortisone administration the pressor response to all concentrations of nor-epinephrine was consistently greater than that found before treatment. The resting pulse rate on cortisone therapy was notably lower (approximately 65 to 70) than before any therapy was administered (approximately 95 to 100).

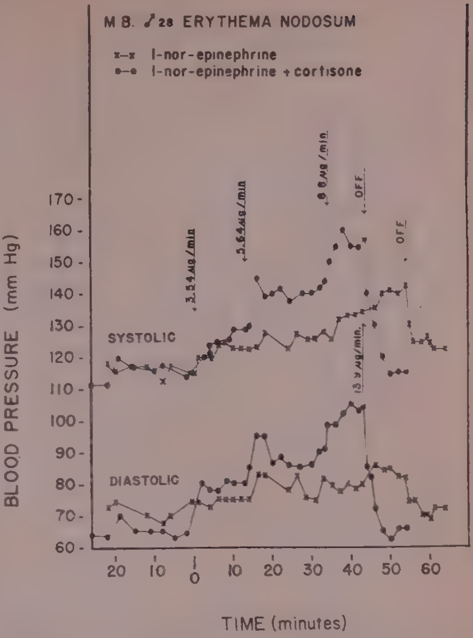

FIG. 1. Case M.B. Blood pressure response to intravenous infusion of varying concentrations of nor-epinephrine before (x———x) and during (●———●) oral cortisone.

Case 2, T.S.P., a 35-year-old woman, BIH No. 18755 was admitted for ACTH therapy during an acute exacerbation of multiple sclerosis. Studies were performed before, during, and after ACTH therapy as indicated in Table I. No other agents were administered. Six infusions were performed during ACTH therapy; 3, before ACTH and 24 and 72 hours after omission of ACTH. The data (Table I) indicate a striking increase in systolic pressor response to nor-epinephrine during ACTH therapy compared to the response before ACTH was administered and when ACTH was omitted. The resting pulse rates were somewhat lower during ACTH therapy.

Case 3, M.K., a 41-year-old woman, BIH No. M19859 was admitted for ACTH therapy of rheumatoid arthritis of 3 years' duration. Although the clinical diagnosis of scleroderma was considered, the histologic diagnosis from a skin biopsy was senile atrophy (elastosis) of skin. Seven infusions of nor-epinephrine were carried out: 3 prior to ACTH; two, 24 and 100 hours after starting ACTH; one 36 hours after omission of ACTH; and one,

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TABLE I.

Exp.	Protocol	No. of eosinophils per mm^3	Avg systolic blood pressure, mm Hg					
	Rate of infusion L-nor-epinephrine		0	3.5 μg/min	5.7 μg/min	8.8 μg/min	13.9 μg/min	0
I	Control	75	105	109	114	127	143	104
II	ACTH, 90 mg daily		108	123	130	140	154	108
III	ACTH, 180 mg daily for one day		110	117	124	132	143	109
IV	ACTH, 180 mg daily for 4 days		110	131	138	153	165	110
V	ACTH, 180 mg daily for 5 days	16	113	135	142	154	>170	118
VI	ACTH omitted for 24 hr	464	110	116	119	125	138	
VII	ACTH omitted for 72 hr		109	114	122	131	151*	
VIII	ACTH, 180 mg daily for 4 days	6	108	120	129	142	158	
IX	ACTH, 180 mg daily for 5 days		109	132	144	154	165	
				Avg pulse rate, beats per min				
	On ACTH		80	71	69	67	65	
	Off ”		95	84	78	71	68	

* Distended bladder.

48 hours after administration of cortisone. The data (Fig. 2) demonstrate the striking difference between the pressor response to nor-epinephrine during ACTH therapy as compared to the response to 3 infusions performed prior to ACTH and one performed 36 hours after omission of ACTH. Subsequently cortisone, 50 mg every 8 hours was administered by mouth for 48 hours and the experiment was repeated. The systolic pressor response to nor-epinephrine was notably greater than during the control period and as great as during ACTH administration. This was true not only when the magnitude of the rise was expressed as mm of mercury but also when the rise was expressed as a per cent of the resting blood pressure. The diastolic blood pressure changes were similar to the systolic but not as striking. The resting blood pressure on ACTH and cortisone was higher than the resting value obtained during the pretreatment period.

Discussion. In previous studies of the pressor response to epinephrine and nor-epinephrine a variety of methods have been used for the administration of these agents. This may, in fact, explain the variable results obtained(12). The validity of conclusions drawn from our data is predicated upon the reliability and reproducibility of the results obtained with the technic we have utilized. In Case 3 (Fig. 2) 4 infusions without ACTH, 2 infusions during ACTH therapy and one infusion during cortisone therapy were performed. The preinfusion systolic blood pressure in 4 control experiments ranged from 96 to 104 mm of mercury and averaged 101 mm of mercury. During the infusion of nor-epinephrine in the same experiments the means of the systolic blood pressure ranged from 126 to 132 mm of mercury and averaged 129 mm. The findings during ACTH therapy are in striking contrast; the mean systolic resting blood pressures were 108 and 112 mm and mean systolic blood pressures during nor-epinephrine infusions were 158 and 161 mm. Similarly, of the 9 studies performed in Case 2, there was no overlap (except in infusion No. 7), between the systolic blood pressure response to l-nor-epinephrine without ACTH and that observed during ACTH therapy. In period 5 of the 7th infusion in this case an unexpectedly high systolic blood pressure was observed; the patient was suffering considerable discomfort from a distended bladder. Additional studies in other patients of the pressor response to epinephrine and nor-epinephrine have also demonstrated the reproducibility of the blood pressure response during successive infusions in the same patient on

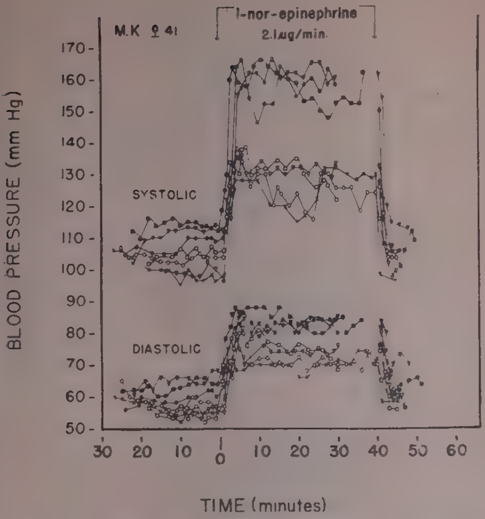

FIG. 2. Case M.K. Pressor response to intravenous infusions of 2.1 µg/min of nor-epinephrine. Open symbols represent observations obtained prior to ACTH or after its omission. Solid symbols represent observations made during ACTH (●——● and ▲——▲) and during oral cortisone (■——■) administration.

different days. These results indicate that variability of technic or variable response of the patient can be eliminated as the basis for the altered sensitivity to nor-epinephrine.

The present data are consistent with the hypothesis that ACTH increases the pressor response to nor-epinephrine by the release of C-11 oxysteroids from the adrenal cortex. Previous observations by others of increased resting blood pressure in patients on DCA therapy alone and the observation that DCA potentiated the response to nor-epinephrine (13) suggested the possibility that ACTH had stimulated DCA production. It is noteworthy that both case 2 and case 3 gained weight during ACTH therapy. However, cortisone alone (Case 3) is at least as effective as ACTH in increasing sensitivity to nor-epinephrine.

The mechanism of action on the cardiovascular system of the C-11 steroids requires further study. The data of Fritz and Levine (15,16) indicate that C-11 oxysteroids act locally on the small splanchnic vessels in animals by sensitizing them to the action of nor-epinephrine. The observations of Brust *et al.*(11) demonstrating the effect of ACTH and cortisone in counteracting the usual depressor effect of TEAC is also consistent with a direct effect (rather than one of reflex origin) on the vasculature. The hypothesis that ACTH and cortisone sensitize the vasculature of man to endogenous nor-epinephrine may explain the elevation of the resting blood pressure during ACTH and cortisone therapy which had been previously noted by others and has also been observed in this study. Similarly, the hypertension of Cushing's disease, previously without adequate explanation, may result from sensitization by increased amounts of C-11 oxysteroids to endogenously formed nor-epinephrine. It is of interest that Goldenberg has reported increased sensitivity to nor-epinephrine in patients with essential hypertension(17). However, no conclusions can be drawn at the present time concerning the relationship between the altered sensitivity to nor-epinephrine demonstrated here in normotensive patients and the genesis of essential hypertension.

Summary. 1. The pressor response to an intravenous infusion of 2.1-13.9 µg per minute of l-nor-epinephrine was studied, using a constant rate infusion apparatus, in 3 patients before and during the administration of ACTH and cortisone. 2. Striking potentiation of the blood pressure response to nor-epinephrine was noted within 24 hours after administration of 90-180 mg ACTH daily or 150-200 mg cortisone daily. The potentiating effect disappeared within 36 hours after the omission of these agents.

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Histochemical Localization of Ribonucleoproteins by Alkaline Hydrolysis.* (18963)

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Several methods for the histochemical identification and localization of ribonucleic acid (RNA) and its differentiation from desoxyribonucleic acid (DNA) are now available. They fall into 3 categories: (1) The utilization of the specific ultraviolet absorption of nucleoproteins as described by Caspersson (1,2); (2) The direct specific staining of nucleoproteins by technics such as the Feulgen-Schiff method for the detection of DNA and Turchini's(3) stain for the detection of both DNA and RNA. (3) The use of substances which depolymerize or alter the nucleic acids so that their basophilic staining properties are lost.

Caspersson's ultraviolet absorption methods have been discussed extensively. They need not be considered further in this connection. The Feulgen-Schiff reaction is the most widely accepted method for the demonstration of DNA. Turchini's technic, which includes the use of the dyestuffs 9-phenyl (or methyl) -2,6,7-trihydroxy-3-fluorone, stains RNA yellow-pink and DNA blue-violet. This method has not been generally accepted. Of the methods in the 3rd category, Brachet's(4) ribonuclease test has been used most widely. The cost of ribonuclease, however, makes this method too expensive for routine use in most laboratories. The activity of the commercially prepared enzyme furthermore is not constant. Other methods which have been reported include the trichloroacetic acid method of Pollister and Ris(5) and the perchloric acid method of Sulkin and Kuntz(6). The former method involves the use of high temperatures which may be objectionable. The latter method yields the most satisfactory results only when frozen sections are used or when small blocks of tissue are treated with the reagent.

In the course of an investigation in which various staining methods have been used following the application of McManus and Cason's(7) acetylation method, it has been observed that a potassium hydroxide bath, such as that used by McManus, abolishes the basophilic staining reaction of the chromidial substance and the nucleoli, in nerve cells without altering the basophilic staining reactions of the nuclei. The present paper embodies the results of a further investigation of this phenomenon.

Methods. Sections of autonomic ganglia of dogs, cats and man were treated with the acetic anhydride-pyridine mixture used by McManus for 40 minutes. This was followed by a 0.1 N KOH bath for 45 minutes. Other sections were placed in a 0.1 N KOH bath for 45 minutes without having been treated with the acetic anhydride-pyridine mixture. The sections were then stained with 0.25% toluidin blue, 1% toluidin blue, Harris hematoxylin and eosin or Mallory's methyline blue-phloxine stain.

The sections treated with the acetic anhydride-pyridine mixture showed no alterations in their basophilic staining properties. Those treated with this reagent followed by a bath of KOH or with KOH alone showed alterations in the basophilic properties, indicating that the 0.1 N KOH is the factor responsible for abolition of the basophilic staining reaction.

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* This investigation was supported by a research grant from the National Heart Institute, U. S. Public Health Service.

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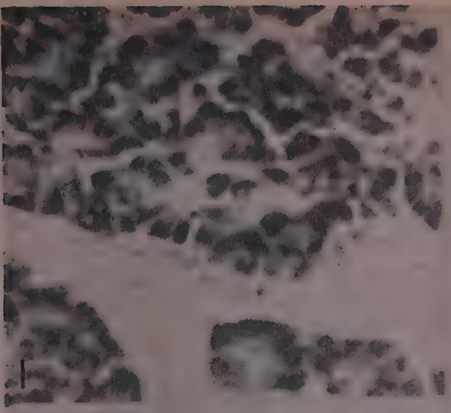

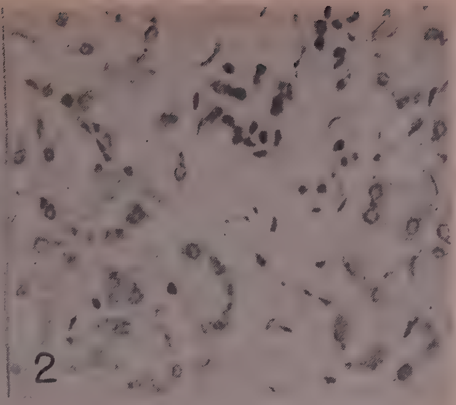

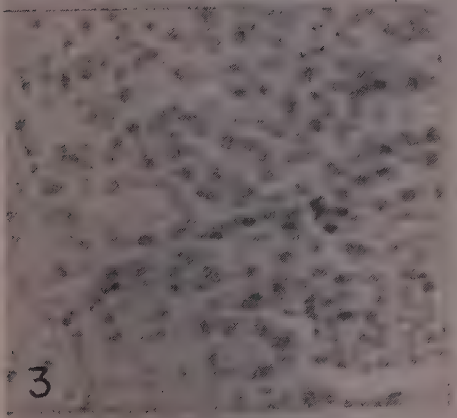

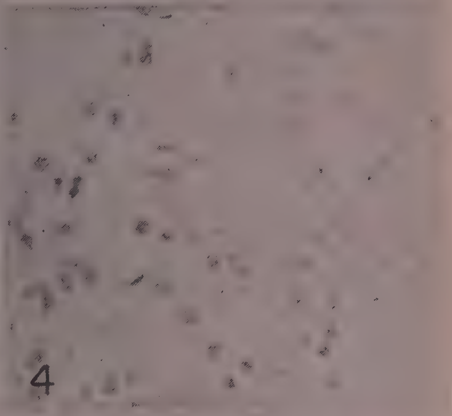

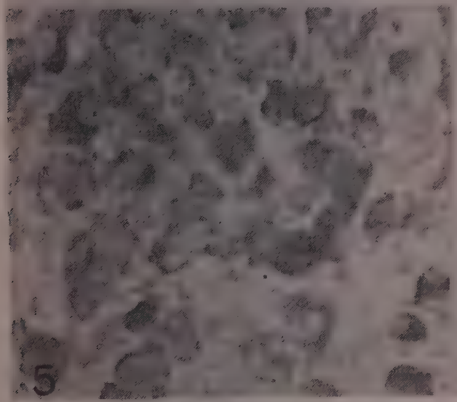

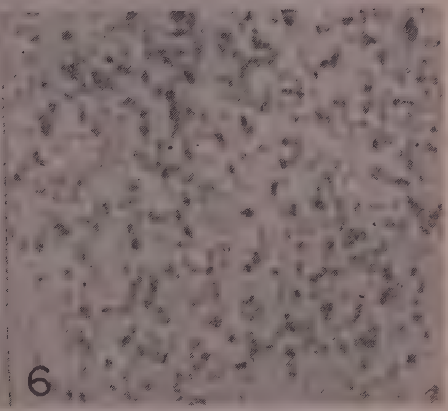

FIG. 1. Section of human parotid gland stained with toluidin blue, ocular 10×, obj. 8 mm.

FIG. 2. Section of pancreas from cat stained with toluidin blue, ocular 10×, obj. 8 mm.

FIG. 3. Section of human parotid from same series of sections as Fig. 1 which was treated with 0.1 N KOH for 45 min preceding the toluidin blue stain, ocular 10×, obj. 8 mm.

FIG. 4. Section of pancreas of cat from same series of sections as Fig. 2 which was treated with 0.1 N KOH for 45 min preceding the toluidin blue stain, ocular 10×, obj. 8 mm.

FIG. 5. Section of autonomic ganglia from 6-week-old dog stained with toluidin blue, ocular 10×, obj. 8 mm.

FIG. 6. Section similar to that in Fig. 5 which had been treated with 0.1 N KOH prior to staining with toluidin blue.

The following procedure has been used successfully on autonomic ganglia of dogs, cats and man, parotid glands of man and pancreas of cats and rats. The tissues were fixed in 10% formalin, Zenker's fixative or 90% alcohol, dehydrated, imbedded and sectioned in the routine manner. Following removal of the paraffin the sections were taken through a degrading series of alcohols and placed in water. One-half of each series of sections was placed in 0.1 N KOH for 45 minutes at room temperature and then thoroughly washed in tap water. The other half of each series of sections was allowed to remain in water during the same time. Both the control and the alkaline treated sections were then stained together in 0.25% or 1% toluidin blue, Harris hematoxylin and eosin or methyline blue-phloxine.

The cytoplasm of the parotid gland cells (Fig. 1) and the acinar cells of the pancreas (Fig. 2) is normally basophilic. Treatment of the sections with ribonuclease prior to staining inhibits the basophilic staining reaction. This indicates that the cytoplasmic basophilia is due to the presence of ribonucleic acid. In all instances, sections of the parotid gland (Fig. 3) and the pancreas (Fig. 4) that were treated with mild alkaline hydrolysis showed no basophilic staining in the cytoplasm. The DNA of the nuclei remained unaltered (Fig. 3 and 4). The basophilic staining reaction of the chromidial substance and the nucleoli of autonomic ganglion cells (Fig. 5), as has been previously reported by Caspersson(1), Gersh and Bodian(8), and others, is due to the presence of RNA. This reaction was inhibited by treatment of the sections with 0.1 N KOH (Fig. 6).

Conclusions. The alkaline hydrolysis method described above for the histochemical localization of RNA and its differentiation from DNA is a simple and convenient method for general use. It is apparently also as reliable as any procedure previously reported.

The differential hydrolysis of RNA and DNA probably is due to the position of the phosphoric acid residues connecting the nucleotides. Because of the greater resistance of the DNA to alkaline hydrolysis as compared to the resistance of RNA, Levene and Tipson(9) have advanced the opinion that the hydroxyls of carbons 3 and 5 of neighboring desoxyribose units of DNA are connected. In RNA, the hydroxyls of carbons 2 and 3 of the ribose units probably are involved. The data obtained in the present study afford further evidence of the resistance of DNA to mild alkaline hydrolysis.

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Comparative Effect of Tryptophan, Niacin, and Nicotinamide in Forming Liver Pyridine Nucleotides.* (18964)

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The ability of tryptophan to replace niacin in the diet of the rat was first demonstrated by Krehl *et al.*(1), who used growth as a criterion of activity. More recent studies in our laboratory indicate that tryptophan and niacin, when incorporated in the ration in equimolar amounts, except at very high levels,

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Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation; and by a grant supplied by the Nutrition Foundation, Inc., New York.

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TABLE I. Composition of Ration.

	%
Sucrose	49.2
Gelatin	40
Sulfasuxidine	1.5
Salts IV	4
DL-methionine	.3
Corn oil	5
Thiamine • HCl	.20 mg
Riboflavin	.30 mg
Pyridoxine • HCl	.25 mg
Calcium pantothenate	2 mg
Biotin	.01 mg
Folic acid	.02 mg
Inositol	10 mg
Choline • Cl	200 mg

Fat soluble vitamins supplied as fortified Haliver Oil, 2 drops per week per rat.

result in about the same production of liver pyridine nucleotides(2). At the levels normally fed in rations tryptophan serves as a physiologically more important precursor of tissue pyridine nucleotides than does niacin(3). The present report is a study on the rate and extent to which single administered doses of niacin, niacinamide and tryptophan are converted to liver pyridine nucleotides in animals depleted of these nucleotides by dietary means.

Experimental. Male Sprague-Dawley rats (200-250 g) were maintained on the ration indicated in Table I. When fed at a 40% level gelatin meets the requirements for all the essential amino acids except methionine and tryptophan. With a methionine supplement 40% gelatin serves as an inexpensive source of amino acids that is complete except for tryptophan. The water-soluble vitamins were fed at the usually prescribed levels (Table I) with the exceptions that niacin was omitted and choline fed at twice the normal level. The higher level of choline was included to prevent depletion of methyl groups when large amounts of niacin or its precursors are fed(4). Sulfasuxidine was added to minimize any synthesis of tryptophan or niacin by the intestinal flora. This ration is biologically complete except for the absence of tryptophan and niacin. The rats were fed this incomplete ration *ad libitum* for about 6 weeks to deplete their tissues of pyridine nucleotides. When analyses indicated that the liver pyridine nucleotides had decreased to about one-half the normal value the animals were utilized for the following experimental work.

Separate 0.0203 molar solutions of niacin, niacinamide, and DL-tryptophan were prepared. The tryptophan was dissolved with aid of dilute sodium hydroxide. Two ml per 100 g body weight of the appropriate solution was fed by tube directly into the stomach of the rat. This volume supplied the equivalent of 5 mg niacin per 100 g body weight. At the times after the stomach tubing indicated in Fig. 1, the animals were sacrificed, livers removed, frozen in a mixture of dry ice and acetone and analyzed for pyridine nucleotides by the method of Feigelson, Williams, and Elvehjem(5). This method measures the sum of diphospho- and triphosphopyridine nucleotides and this will be referred to simply as liver PN. The results are shown in Fig. 1, where each point represents the mean value of from 2-4 animals. The average deviation from the mean is ± 66.

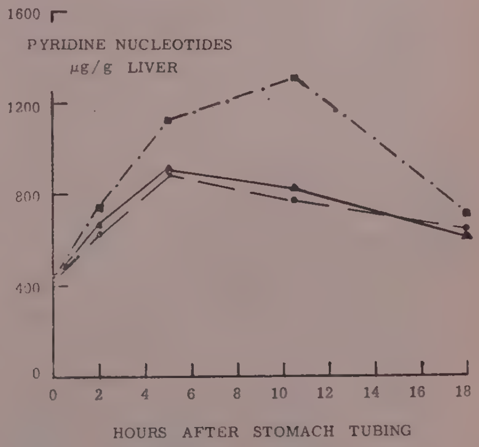

FIG. 1. The effect of a single dose of niacin, niacinamide, or tryptophan on the pyridine nucleotide level of rat liver. —▲—— niacin, —■—•— niacinamide, —●— — tryptophan.

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TABLE II. Effect of Equimolar Supplements of Niacin, Niacinamide and Tryptophan Upon Liver Pyridine Nucleotide Formation.

No. of animals	Supplement		Avg μg PN/g liver		Range
7	0	111	444	213	424-464
6	70 mg % niacin		657		553-780
6	70.5 mg % niacinamide		565		486-612
6	116 mg % DL-tryptophan		635		526-762

It is apparent that liver PN rapidly increased following the administration of all of the supplements. After 2 hours a definite increase was noted, and the PN level continued to rise to a maximum until 5-10 hours. This was followed by a decrease in PN until at 18 hours the liver PN was almost back to its starting level. Almost identical changes in PN followed the equimolar doses of tryptophan or niacin. The PN level rose to a maximum of 900 μg/g liver 5 hours after supplementation, then decreased slowly until at 18 hours it reached a level of 650 μg/g liver. The response to an equimolar amount of niacinamide, also administered by stomach tube as a single dose, was quite different from that of niacin and tryptophan as shown in the figure. Following niacinamide administration the PN level reached a maximum level of 1310 μg/g liver in 10 hours, which declined sharply to 720 μg/g liver at 18 hours.

To study this phenomenon further more animals were depleted of tissue PN as described above. They were divided into 4 groups and fed the basal ration plus the supplements, indicated in Table II. The supplements of tryptophan, niacin or niacinamide were equimolar and were incorporated directly into the ration. The level of the supplements was chosen so that the rats would consume daily approximately the same amounts of the supplements force-fed in the preceding experiments. The 4 groups of animals were fed the respective supplemented rations *ad lib.* for 2 weeks, and their livers analyzed for PN. The results are shown in Table II. In all supplemented groups there is a significant increase in PN level above the group receiving no supplement. There is no indication that when niacinamide is incorporated into the ration it is superior to either niacin or tryptophan as a precursor of PN.

Discussion. The present study was conducted under conditions of severe tryptophan deficiency and, even under such conditions, tryptophan administration resulted in the same PN formation as equimolar amounts of niacin. The identity of the curves after force-feeding equimolar amounts of niacin and tryptophan (Fig. 1) implies that, at least in the rat, the conversion of tryptophan to PN is not a minor but rather a major metabolic route of tryptophan metabolism. The apparently greater efficiency of a single dose of niacinamide as a precursor of PN as compared to niacin or tryptophan might be explained by the fact that niacinamide does not have to be amidated before forming PN, whereas niacin does.

The return of the liver PN level to almost the original value after 18 hours indicates a relatively short lifetime for the PN molecule and an inefficient conservation of the niacinamide residue from the catabolized PN.

Summary. Changes in the level of liver pyridine nucleotides (PN) of rats were studied at various times following the administration by stomach tube of equimolar amounts of tryptophan, niacin, or niacinamide. Tryptophan was observed to be equally effective as a PN precursor as equimolar amounts of niacin, whereas niacinamide produced significantly higher levels of liver PN than either tryptophan or niacin. When incorporated directly into the ration, however, niacin and tryptophan were again equivalent as PN precursors whereas, under these conditions, niacianamide was a slightly less efficient PN precursor than niacin or tryptophan.

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Amyloidosis and Hypertension in Cholesterol-Fed Rabbits.* (18965)

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Amyloidosis was observed at necropsy of rabbits fed cholesterol for long periods. Deposits of similar structure and distribution, but which did not take specific stains (paramyloid), were found in other cholesterol-fed rabbits. In this communication the histological findings, relevant biochemical observations and changes in blood pressure are reported.

Technic. Eighteen rabbits 6 to 8 weeks old were fed a basic diet of Rockland Farm pellets for periods varying between 12 and 104 weeks. The cholesterol ration for each week was dissolved in ether and poured over the pellets. Following evaporation of the ether, the rabbits readily ate the cholesterol-coated pellets. Chemical and electrophoretic determinations were made by technics outlined previously(1). Mean arterial pressure was determined at weekly intervals by direct puncture of the central ear artery using a 20-gauge needle attached to a mercury manometer. In 138 determinations on normal control rabbits the mean value was 88.1 mm Hg, with a standard deviation of 11.7 mm Hg. No control animal had a mean arterial pressure of more than 120 mm Hg.

Incidence of amyloidosis and paramyloidosis. Of 18 rabbits fed cholesterol for 3 months or longer, 7 or 39%, developed amyloidosis or paramyloidosis. The weekly cholesterol ration in 6 of the 7 animals was 3 g and in the other 20 g. The shortest period in which deposits developed was 12 weeks. However, 2 rabbits, fed cholesterol for 100 and 104 weeks, respectively, did not exhibit amyloidosis or paramyloidosis at autopsy. The weekly cholesterol dosage, duration of feedings and occurrence of amyloidosis or paramyloidosis are shown in Table I.

TABLE I. Occurrence of Amyloidosis in Cholesterol-Fed Rabbits.

Rabbit No.	Cholesterol, g per wk	Duration of feeding (wk)	Amyloidosis or paramyloidosis
1	20	12	+
2	20	16	—
3	20	20	—
4	20	28	—
5	3	29	+
6	20	32	—
7	3	36	+
8	3	36	—
9	3	44	—
10	3	44	—
11	3	48	—
12	3	48	+
13	3	53	+
14	3	56	—
15	3	73	+
16	3	82	+
17	3 20	60 40 } 100	—
18	3	104	—

Histologic findings. Amyloid and paramyloid deposits were found only in the kidney, liver and spleen. The earliest glomerular lesions consisted of small, ovoid, homogeneous anuclear deposits which stained pink in the hematoxylin-eosin preparations. These were found in many glomeruli, but only portions of glomeruli were affected. In the more advanced cases, all the glomeruli were involved. The glomeruli were markedly enlarged and their capillaries often almost completely obliterated by the eosinophilic, homogeneous substance. Bowman's space was obliterated in some of the glomeruli. Generally, the homogeneous substance stained blue with azocarmine, light tan in the van Gieson and red in the Gram preparations. Appreciable amounts of lipid within the glomeruli were demonstrated with Sudan IV in 2 of the 7 rabbits. Paraffin and frozen sections of the kidneys were stained with Congo red and methyl violet in 6 of the 7 animals. Results indicative of amyloid (bright red with Congo red, metachromasia with methyl violet) were obtained in 2 instances with both stains and

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* Aided by grants from Mr. Percy Uris and Mr. Louis Adler.

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TABLE II. Distribution and Staining Reactions of Amyloid and Paramyloid Deposits.

Rabbit	Glomerular deposits		Splenic deposits		Hepatic deposits	
No.	Congo red	Methyl violet	Congo red	Methyl violet	Congo red	Methyl violet
1	—	—	+	—	0	0
5	+	+	—	+	—	+
7	—	—	+	—	—	—
12	—	—	0	0	0	0
15	+	—	—	—	0	0
16	+	+	—	+	+	—

+ Amyloid stain positive. — Amyloid stain negative. 0 No deposit observed.

in another with Congo red alone. More brilliant staining of the amyloid deposits was achieved in the frozen sections. The collecting tubules were widely distended with casts, which compressed and flattened the tubular epithelium. The casts were pink in hematoxylin-eosin preparations, green with van Gieson and blue with Gram's stain. Generally the casts were scarlet with azocarmine but some, especially in kidneys with older lesions, were blue. In no instance did the casts react positively to either Congo red or methyl violet. The epithelial cells of the convoluted tubules contained large hyaline droplets, best defined when the autopsy was performed very promptly after death. These appeared deep red in the azocarmine, blue in the Gram, and dark brown in the van Gieson preparations. The droplets did not give a positive reaction with either amyloid stain. The renal arteries were normal. In the spleen the amyloid was deposited in the pulp without involving the center of the follicles or the arteries. The hepatic amyloid was not extensive and was largely confined to the midzone of the lobules. The results of the Congo red and methyl violet stains in the 6 amyloid or paramyloid rabbits in which they were carried out are shown in Table II.

Urinary changes. Tests for urinary protein were done in 12 of the 18 rabbits fed cholesterol. Of these, 8 were without amyloid or paramyloid. None of the 8 had proteinuria. Of the 4 rabbits with amyloid or paramyloid, 2 had proteinuria. Electrophoretic study of the urine of these 2 rabbits revealed that globulins constituted a high proportion of the urinary protein (Fig. 3).

Changes in blood chemistry. The hyperglobulinemia produced in rabbits by cholesterol feeding has been reported previously(1). Identical changes were observed in the sera of hypercholesteremic rabbits which developed amyloidosis (Fig. 3). All the rabbits had great hypercholesteremia. Monthly determinations of the nonprotein nitrogen of the serum revealed significant nitrogen retention in only one of the animals with renal lesions and that only terminally.

Changes in blood pressure. Blood pressure was measured in 12 of the 18 rabbits. In the amyloid or paramyloid group of 7 rabbits, blood pressure was determined in 5. All rose above the control levels. Hypertension is regarded as present in those animals in which

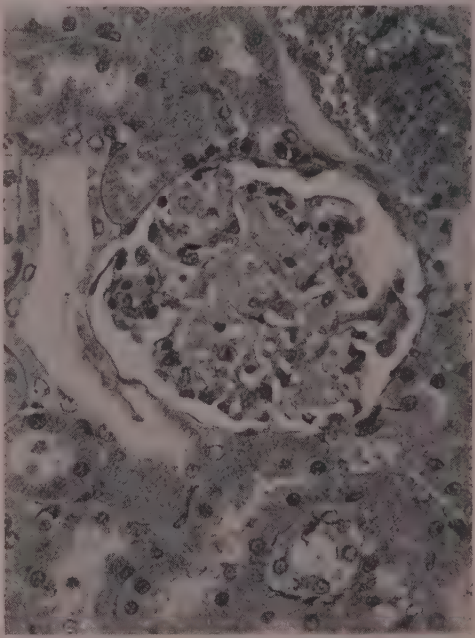

FIG. 1. Rabbit 1. Amyloid in glomerulus of rabbit fed 20 g of cholesterol per week for 12 weeks (hematoxylin and eosin).

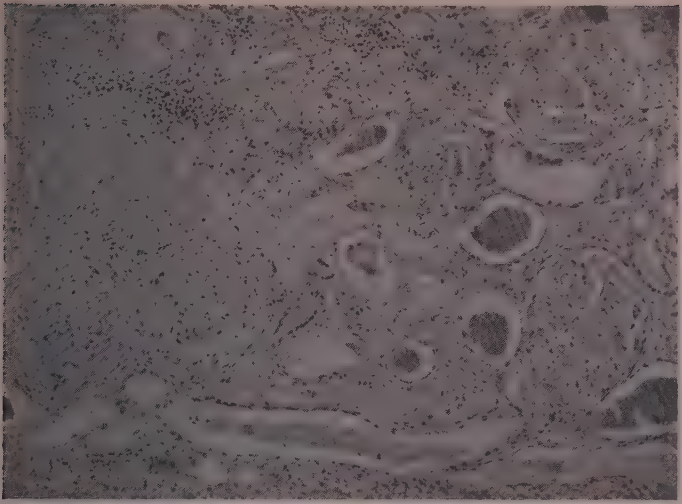

FIG. 2. Rabbit 15. Far advanced amyloidosis of the kidney in a rabbit fed 3 g of cholesterol per week for 73 weeks (hematoxylin and eosin).

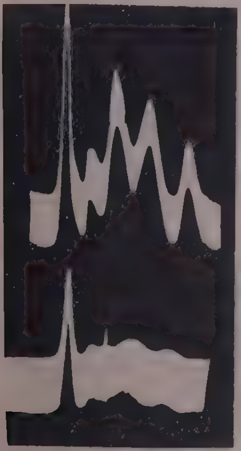

FIG. 3. Rabbit 5. Electrophoretic patterns of serum (above) and urine (below) after ingestion of 3 g of cholesterol weekly for 29 weeks. Necropsy revealed renal amyloidosis. Relative percentages of protein components:

	Serum	Urine
Albumin	24	48.7
α_1 globulin	7.2	16.5
α_2 "	29.4	24.8
β "	27.5	10.5
γ "	12	—

the arterial pressure rose above the mean of the 138 normal control measurements plus 3 times the standard deviation, *i.e.*, 123 mm Hg. This condition was fulfilled in 4 of the 5 animals with amyloidosis.

Of the 11 animals in the non-amyloid group, blood pressure was measured in 7. One of these, a rabbit fed 3 g of cholesterol weekly for 104 weeks, maintained a mean pressure between 140 and 205 mm Hg for over 30 weeks, with an average reading of 175 mm Hg. This was the most severe hypertension observed in the entire series. Of the 6 remaining rabbits in the non-amyloid group in which the blood pressure was measured, 3 showed a moderate rise above the control values. In none, however, was a level of 123 mm Hg reached.

Discussion. Of 18 rabbits fed 3 to 20 g of cholesterol weekly for 12 to 104 weeks, 7 developed amyloidosis or paramyloidosis. In necropsies of 38 rabbits kept under the same conditions and with identical diets, but which were not given cholesterol, these lesions were not observed. Of the 7 rabbits with amyloidosis or paramyloidosis, 3 manifested infections at necropsy (1 pneumonitis, 1 subcutaneous abscess, 1 cellulitis of a paw). Two other rabbits had superficial ulcerations of the soles, attributed to the wire floor of the cages, and which are not uncommon in rabbits kept in such cages in this laboratory. Of the 11 cholesterol-fed rabbits without amyloidosis, 3 had infections (2 ear infections, 1 purulent mediastinal mass) and 3 ulcerations of the soles. Marked atheroma of the aorta and

TABLE III. Mean Arterial Pressure in Rabbits with Renal Amyloidosis and Paramyloidosis.

Rabbit No.	Control B.P. (mm Hg)	Maximum mean experimental B.P. (mm Hg)
1	85	125
5	90	135
7	80	115
15	100	150
16	85	140

other large arteries was present in all the rabbits.

The available evidence does not definitely indicate the mechanism through which cholesterol feeding in rabbits sometimes results in amyloidosis. However, a significant role is perhaps to be attributed to the hyperglobulinemia which develops. Hyperglobulinemia has repeatedly been demonstrated in various clinical and experimental circumstances in which amyloidosis occurs. The lesions in the kidneys of our animals closely resemble those produced by Sussman and Freed(2) by injection of a 10% globulin solution (rabbit antipneumococcic serum). Cholesterol feeding would appear to be another method by which amyloidosis can be produced through the intermediacy of hyperglobulinemia.

Since amyloidosis does not constantly result from hyperglobulinemia in any of the conditions studied (human and experimental tuberculosis, multiple myeloma, casein feeding, injection of bacteria or their products, etc.), other pathogenetic factors must also be involved. These are as obscure in the amyloidosis that develops in some rabbits fed cholesterol as in other forms of amyloidosis.

Of the 5 animals with amyloidosis in which blood pressure was measured, 4 had hypertension. Of 7 rabbits fed cholesterol which did not develop amyloidosis, only one had unequivocal hypertension. However, this animal had the highest mean pressure (175 mm Hg average for over 30 weeks) observed. That feeding cholesterol to rabbits results in hypertension has been both affirmed(3) and denied(4). Our data show that cholesterol feeding does result in hypertension in some rabbits. Though the findings indicate that hypertension is more apt to result from cholesterol feeding in rabbits if amyloidosis is produced, the blood presure may also become elevated in the absence of amyloid. Renal amyloidosis is thus not a necessary intermediary in the pathogenesis of hypertension produced in rabbits by feeding cholesterol. Whether the extensive atherosclerosis of the aorta and some of the other arteries present in all the animals affects the blood pressure remains to be established.

The finding of a high percentage of globulins in the proteinuria of rabbits in which amyloidosis was produced by cholesterol feeding harmonizes with the long known fact that the proportion of globulin in the urinary protein of human renal amyloidosis tends to be high.

Summary. Of 18 rabbits fed cholesterol for 12 to 104 weeks, 7 developed amyloidosis or paramyloidosis. These rabbits had hyperglobulinemia and their urinary protein contained a high percentage of globulins. Hypertension developed in 4 of 5 rabbits with amyloidosis and in 1 of 7 without such deposits.

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Effect of Tocopherol Feeding on the Composition of Turkey Tissues.* (18966)

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In vivo studies of the antioxidant value of the tocopherols have been relatively few. Prevention of peroxide accumulation in the fat of rats by feeding tocopherols has been shown(1,2) and the conclusion drawn that the normal source of tissue antioxidants is the tocopherol of the feed. Similar experiments on hog fat have indicated similar relationships between keeping quality of the fat and the presence of tocopherols and unsaturated fatty acid(3). However, an attempt to reduce rate of rancidification of pork fat by feeding or injecting tocopherols before slaughter proved largely unsuccessful(4). Major and Watts(5) obtained protection from rancidity in rabbit meat by feeding or injecting 1 or 11.1 mg per day of mixed tocopherols for an unspecified period. When 400 mg of mixed tocopherols or tocopherol phosphate was injected in 2 doses during the 3 weeks previous to sacrifice into rabbits fed a natural diet no protection against rancidity of the meat resulted. Carpenter and Lundberg(6) in contrast with Watts, Cunha, and Major(4) found the induction period for oxygen absorption much shorter in fat from hogs which had not received supplementary tocopherols than in those which had received a total of 31 g in 12 weeks.

Tocopherol content of tissues following feeding of tocopherols has been determined only in the rat(7) and hog(8). Bioassay used by Mason(7) indicated wide distribution of tocopherols in rat tissues with liver deposits most significantly changed by increases in intake. Only a small fraction of the excessive amounts ingested was found in the tissues, the total amount found after 2 to 4 months feeding being only 3 times the daily dose. This was confirmed by Hines and Mattill(9), who indicated large concentrations of tocopherols in the feces of rats given supplements of the vitamin. The muscle as well as the liver and other viscera of rats given graded doses of tocopherol was found to increase moderately in tocopherol content from 4.8 mg per kg on vit. E deficient diet to 7.5 and 11.9 with increasing levels of intake(7). Rabbits were found to store less tocopherol in the liver in comparison with the muscle than did rats(9).

No studies of tocopherol content and keeping qualities of poultry tissues appear to have been recorded. Since the development of rancidity in turkey fat and meat is usually rapid even under the best feeding and storage conditions, it appeared worth while to measure the effect of graded doses of tocopherols upon the production of peroxides and the development of rancidity in stored turkey carcasses. The tocopherol content of the tissues was determined simultaneously.

Experimental methods. Two lots of turkey hens of the Bronze strain were examined. The method of feeding, slaughtering and storing has been previously described(10). There

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* The cooperation of V. S. Asmundsen, University of California at Davis in the production of the turkeys is gratefully acknowledged, also of the Western Regional Research Laboratory at Albany, Calif. for storage of the birds, of Marion Olmstead, Jayne H. Prentice and Janet Dwight for technical assistance. Assistance was also given by the Poultry and Egg National Board.

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were 12 birds in the first series, averaging 6 kg in weight when slaughtered at 34 weeks of age, and 16 birds in the second series averaging 6.5 kg when slaughtered at 36 weeks of age.

The mash fed contained alfalfa meal 7.5, wheat 32.5, wheat bran 10.0, corn 7.0, barley 25.0, fish meal 5.0, soybean meal 10.0, limestone and bone meal 2.0, salt 0.5, fish oil 0.5, and manganese sulfate 0.025. This ration was calculated to contain about 1.8 mg % tocopherols of which 0.9 mg % was α-tocopherol on the basis of the tocopherol analyses of Harris *et al.*(11). The maximum intake of α-tocopherol of the birds from the basal ration was not more than 1.5 mg per day and per kg per day not more than 0.25 mg, if this calculation is correct. No signs of vit. E deficiency were seen.

Tocopherols were added to this feed in the form of a concentrate containing per g 50 mg mixed tocopherols,† about one-half α and one-half mixed β and γ, chiefly γ. In the first series 4 groups of birds received for 5 weeks respectively per 100 g ration, 100 mg tocopherols, 10 mg tocopherols, none, and a vit. E-depleted feed. The latter was prepared by treatment with 1% ferric chloride solution. In the second series of 5 groups, 3 received the ration containing 20% of the tocopherol concentrate, or 1 g mixed tocopherols per 100 g ration, for 21, 7, and 2.5 days, respectively. The fourth group received 50% of the ration as the concentrate or 2 g mixed tocopherols per 100 g ration for 2.5 days, and the fifth group received the basal ration only.

At the end of various storage periods the carcasses were cut in half, one-half of each was roasted, judged for palatability and sampled for thiamine, riboflavin and niacin contents. The other half was used uncooked for determination of water, fat, tocopherols, peroxides, thiamine, riboflavin and niacin (10).

The tocopherol method used was a modification of the iron-α, α'-bipyridine color method of Hines and Mattill(9). Carotene was found to remain in the florisil treated extract of the livers but was removed successfully by treatment with concentrated (85%) sulfuric acid, (2 ml per 10 ml petroleum ether extract). The recovery of added tocopherols under these conditions when added cholesterol, carotene and vit. A were present in the tissue extracts, was 94 to 100%.

This method was unsatisfactory for the determination of tocopherols in fat. When only 30% of added tocopherol could be recovered from abdominal fat samples the analysis of fat for tocopherols was abandoned. The apparent loss of tocopherol occurred whether the tocopherol was added before or after extraction of the fat by the method of Chipault *et al.*(3).

The measure of fat rancidity was peroxide concentration measured by the method of French, Olcott, and Mattill(12). The Kreis test was also employed at first but since it was found that small amounts of tocopherol, 30 mg per g fat, reacted positively with the phloroglucinol reagent this test was eliminated.

The livers were analyzed for vit. A and carotene by the usual antimony trichloride and colorimetric procedures.

Bioassay of the liver and leg muscle extracts was carried out in order to estimate the character of the tocopherols measured since no such comparison had been previously made. Both depleted virgin and resorption female rats used as suggested by Mason(13) were found to respond similarly to tocopherol dosing. Only rats under 6½ months of age were used. The international standard for vit. E, racemic α-tocopherol acetate, was used at 3 levels, 1.2, 1.0 and 0.8 mg, to establish the mean fertility dose. Pooled liver and leg muscle samples were fed in 5 days after the mating in amounts calculated to provide 1.0 mg tocopherol as determined by the chemical method. The animals were allowed to deliver the litters instead of being sacrificed on the

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TABLE I. Tocopherol and Peroxide Content of Turkey Tissues As Affected by Tocopherol Feeding and Storage.

			Tocopherol feeding				Acceptability			Peroxides in mEq./kg		Tocopherols in mg per kg					
								Rancidity									
Series	Group	Storage, mo	Time, days	Total amt, g	Level in feed, %	Bird No.	Rating	Thigh	Fat	Skin fat	Abdominal fat	Liver	Heart	Gizzard	Leg	Breast	Liver vitamin A, $\mu g/g$
1	1	3	35	4.2	.1	1	Excel†	none	none	.2	.5	159					
	2		35	.4	.01	4	”	”	”	.1	.1	155					
	3		35	0	0	7	Fair	ran‡	ran‡	1.5	4.5	135					
	4		35*	0	0	10	Good	none	none	2.1	7.2	23					
	1	6	35	4.2	.1	2	Excel	”	”	.7	2.1	101			35	14	
	2		35	.4	.01	5	Good	ran	ran	1.5	5.4	67			22	9	
	3		35	0	0	8	Fair	none	none	4.3	13.4	42			12	9	
	4		35*	0	0	11	Poor	ran	ran	3.9	7.4	49			13	9	
	1	9	35	4.2	.1	3	Excel	none	none	1	4.8	80	107	45	24	11	4
	2		35	.4	.01	6	Poor	ran	ran	1.8	5.9	59	60	26	15	11	7
	3		35	0	0	9	”	none	”	2	6.7	52	52	19	13	10	4
	4		35*	0	0	12	”	ran	”	7.3	19.4	39	68	21	12	9	0
2	1	0	21	32.9	1	13	Excel	none	none	0	.7	218			50	25	
	2		7	11.3	1	17	”	”	”	0	1.2	119			43	10	
	4		2.5	8.7	2	25	Good	”	”	.3	1.9	153			13	9	
	3		2.5	3.4	1	21	”	ran	”	.4	.5	94			12	9	
	5		0	0	0	27	”	”	ran	.6	2	50			12	8	
	1	3	21	32.9	1	14	Excel	none	none	.9	2.2	210	80	26	27	11	50
	2		7	11.3	1	18	Good	”	”	.9	3.1	89	58	16	13	10	101
	4		2.5	8.7	2	26	”	”	”	2.7	5.4	87	28	16	11	9	37
	3		2.5	3.4	1	22	”	”	”	1.6	3.4	98	57	15	12	7	56
	5		0	0	0	28	Poor	ran	ran	1.1	4.6	40	63	18	11	8	72
	1	5	21	32.9	1	15	Excel	none	none	.7	5.5	275	133	28	22	10	44
	2		7	11.3	1	19	Good	”	”	1	1.6	108	74	18	10	9	21
	3		2.5	3.4	1	23	Poor	ran	ran	1	5.1	125	80	21	12	10	90
	1	5	21	32.9	1	16	Good	none	none	.6	1.5	227	145	33	17	9	4
	2		7	11.3	1	20	Poor	ran	ran	1	2.9	86	84	31	15	11	39
	3		2.5	3.4	1	24	”	”	”	1	7.4	85	86	21	10	9	29

* Fed a vit. E depleted diet for 35 days before slaughter.
† Excel = excellent; ‡ ran = rancid.

16th day, as recommended by Mason. Wide mesh screens were used in the delivery cages so that the young were dropped out of reach of the mother. Total implantation sites were counted and the number and weight of live and dead fetuses recorded. Fertility was judged by the per cent of litters containing live young and of implantations yielding live young(14). Occurrence of pseudopregnancies and first litter fertility was negligible.

Results. Tocopherol content of tissues. The tocopherol content of liver, heart, breast, gizzard and leg muscle as determined chemically is shown in Table I along with the peroxide numbers of skin and abdominal fat and the acceptability to a panel of judges of the meat and fat. The thigh muscle was also analyzed for tocopherols in a few birds, but since these values were found to be nearly the same as those for leg muscles they are not included in the table.

As shown by Mason(7) for the rat, the liver store was the best index of the tocopherol status of these birds since with increased intakes the liver tocopherol stores were in all cases increased. The maximum liver storage appeared to be limited, however, to about 225 mg per kg fresh tissue, no matter how great the intake. The minimum level was 23 to 52 as found in the livers of birds which received either no added tocopherols or a vit. E-depleted diet for 35 days before slaughter.

The heart tocopherol content, determined in only 15 birds, was usually somewhat smaller than that of the liver, but was less affected by the intake. The gizzards of these same 15 birds were found to contain about the same amount of tocopherols as the leg muscle with only small variations attributable to intake. Leg muscles showed definitely increased tocopherol content in the birds which had received the largest intakes but breast muscles had a fairly constant and minimum content of 8 to 11 mg per kg.

The thiamine, riboflavin and niacin values of the tissues of these birds as previously reported(10) show certain interesting correlations with the tocopherol concentrations. In all cases the tocopherol figures parallel roughly those of riboflavin and generally also of thiamine but not of niacin. Thus the heart had high concentrations of thiamine, riboflavin and tocopherol as compared with other tissues, but not of niacin. The liver was richest of all in riboflavin and tocopherol, the gizzard and leg muscles well endowed with riboflavin and tocopherol but poor in niacin, the breast muscle rich in niacin, poor in thiamine, riboflavin and tocopherol. Some parallelism in metabolic function of riboflavin and possibly also of thiamine with that of the tocopherols may be cautiously suggested.

Effect of storage on tocopherol of tissues. Except in the group in series 2 fed the largest amount of tocopherols the tocopherol content of the liver decreased with storage. This tendency is not evident in the limited number of cases in which the hearts and gizzards were examined, nor did leg and breast muscles show much change.

Rancidity judged organoleptically. A panel of 6 judged the cooked meat of breast, thigh and leg and skin and fat from the back of each of the birds. In no case was the breast meat found rancid. The thigh meat and fat were judged rancid in only 1 of 10 birds, which received any added tocopherols when examined at zero or 3 months storage, but 3 of 3 samples were judged rancid in those which had received no added tocopherols. After 5 or 6 months storage 3 of 6 birds which had received tocopherols and 1 of 2 which had not, exhibited some rancidity. After 9 months, only 1 of 4 birds, that which received the most tocopherol, had no rancidity. The general acceptability of the meat paralleled the amount of rancidity detected.

The peroxide number. The peroxide content of the skin and abdominal fat was generally greatest in the birds which received the least tocopherol. An exception was seen in the 2 birds of group 4, series 2, which received a total of 8.7 mg tocopherol in 2.5 days before slaughter. The peroxide numbers of the tissues were similar to those of the 2 birds in the same series which had no supplement and greater than those which had received half as much (group 3) tocopherol for 2.5

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14. Kaunitz, H., and Beaver, J. J., *J. Biol. Chem.*, 1946, v166, 205.

TABLE II. Bioassay of Turkey Liver and Leg Muscle for Tocopherol Content.

Tocopherol fed		No. of rats	No. of litters containing live young	No. of implantation sites	Total No. of young	Live litter efficiency, %	Live implantation site efficiency, %
Form	Amt, mg						
α-tocopherol acetate	1.2	11	9	92	79	82	67
”	1	8	6	66	49	75	54
”	.8	7	3	80	51	43	39
Liver	1 *	15	12	100	79	80	63
Leg muscle	1 *	7	4	60	26	57	28
None		20	0		0	0	

* As determined chemically.

days. Perhaps the higher concentration of tocopherols in the feed promoted increased waste in absorption.

The peroxide content of fat under the skin was inversely related to the tocopherol content of the liver in both series. The same relationship held for the abdominal fat with minor differences.

Vitamin A of the liver as affected by tocopherol intake. As shown in Table I, there was no relationship between the amounts of tocopherol and of vit. A in the livers or between the intake of tocopherols and the liver vit. A. Since the tocopherol feeding periods were short compared with the age of the birds the liver storage prior to the period of tocopherol supplementation may have varied widely. Some loss in liver vit. A may have occurred in storage as indicated by the low levels found in the birds of series 1 examined after 9 months frozen storage. No data were collected, however, on birds of this series stored for shorter periods. The carotene or "yellow values" for the livers were fairly constant and bore no obvious relationship to the vit. A values or to the levels of tocopherol fed. These results differ from those of the better controlled experiments of Harris *et al.*(15) with rats.

Character of the tissue tocopherol. The mean fertility dose of the rats used was close to 0.9 mg and the turkey liver and leg muscle samples fed at the calculated 1.0 mg level produced litters to correspond with this expectation (Table II). Apparently the tocopherols measured in these 2 tissues were chiefly if not wholly α-tocopherol.

Discussion. Total retention of the added tocopherols in all cases represented a very small fraction of the intake. In a representative of the group fed the most vitamin, the total retention in the organs analyzed and in the remainder of the edible carcass, assuming an average of leg and breast muscle tocopherol values for the whole carcass was only 76 mg out of the total intake of 32.9 g, of which approximately 16.5 g was α-tocopherol. This excludes any excess concentration of tocopherol in the fat and is therefore probably lower than the true value.

Importance of the time factor in securing tocopherol retention is seen in the superior content of the tissues of the birds fed relatively low levels over 35 days in series 1 as compared with the much larger amount for 7 days in series 2. Some increase in tocopherol of the tissues was secured nevertheless even when the feeding period was restricted to 2.5 days.

Relative increases in tocopherol content obtained by excess intake were 6 times for liver, 2-3 for heart, 2-4 for leg, 1-2 for breast and gizzard. This relatively large ability of the turkey to store vit. E in the liver as compared with the muscles resembles the behavior of the rat rather than the rabbit(9).

The response of vit. E-depleted female rats to administration of turkey liver or leg muscle indicated that the liver tocopherol was probably entirely α-tocopherol, since the calculated mean fertility dose corresponded with that found, but the turkey leg muscle had the value of 0.8 to 0.9 mg instead of the 1 mg obtained by chemical analysis. If the mean fertility dose of these rats was taken as 0.9 mg, the liver contained about 10% more and

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15. Harris, P. L., Kaley, M. W., and Hickman, K. C. D., *J. Biol. Chem.*, 1944, v152, 313.

the muscle about 10% less than the amount measured by the chemical method, which is probably within the limits of error of the methods.

There was little doubt that the rancidity as detected by the judges and indicated by the peroxide numbers was slowed by addition of tocopherols to the diet just previous to slaughter. The shortest period of supplementation, 2.5 days on feed containing either 1 or 2% tocopherols, appeared to afford little protection against rancidity although some increase in tissue tocopherol occurred. The longest period, 35 days at 0.1% addition was effective up to 9 months storage, but the same period of supplementation at the 0.01% level was ineffective. The 21-day feeding at 1% level was most effective of all and the same level maintained for only 7 days nearly as good. The lowest effective level used therefore was 0.1% of the feed for 35 days but at 1% nearly as effective protection was obtained by 7 days feeding.

Major and Watts(5) noted no protection of rabbit meat when 400 mg mixed tocopherols was fed with a natural feed 3 weeks before sacrifice of the animals. The amount and continuity of the dosage may have been insufficient. Our observations confirm the conclusions of Chipault *et al.*(3) and Carpenter and Lundberg(6) as to relationship of tocopherol content and keeping quality of hog fats.

Summary. Mixed tocopherols, about one-half α and the rest chiefly γ, fed to 28 female turkeys for 2.5 to 35 days before slaughter at levels 0.01, 0.1, 1 or 2% of the feed produced measurable increases in the tocopherol content of liver, heart, gizzard and muscle. The 0.1% level fed for 35 days produced the most efficient transfer of tocopherol, but the total amount of the supplement retained in this and the other groups was extremely small. The liver usually contained more tocopherol than any other tissue, 23 to 225 mg per kg fresh tissue, the heart contained 28 to 145, the gizzard 15 to 45, the leg muscle 11 to 50, and the breast muscle 8 to 25. Bioassay of liver and leg muscle indicated the presence of α-tocopherol in amounts within 10% of those obtained by the chemical procedure. No relationship was found between the tocopherol and the vit. A and carotene contents of the 15 livers examined. The peroxide numbers of the fat under the skin and the abdominal fat before and after frozen storage for 3, 5, 6, or 9 months were progressively greater with storage and with decreasing amounts of tissue tocopherol. The acceptability of meat and fat and the detection of rancidity to taste by a panel of judges fell in the same order as the peroxide numbers.

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A Non Adrenal-Mediated Action of ACTH on the Preputial Glands. (18967)

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Various steroids, particularly testoids, stimulate the growth of the preputial glands, both in male and in female rats(1). It has been shown, furthermore, that certain pituitary extracts, which in themselves are almost inactive, greatly augment the preputial gland stimulating effect of certain steroids(2,3). Although these pituitary extracts were rather rich in corticotrophic potency, some of the earlier investigators felt their effect upon the

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* Fellow of the Swiss Academy of Biology and Medicine.

1. Selye, H., Textbook of Endocrinology, *Acta Inc. Med. Publ.*, Montreal, Canada (1949).

2. Noble, R. L., and Collip, J. B., *Endocrinology*, 1941, v29, 943.

3. Selye, H., and Clarke, E., *Rev. Canad. de Biol.*, 1943, v2, 319.

TABLE I. Effect of 6 Days Treatment with ACTH.

Group	Treatment	Body wt, g Initial	Body wt, g Final	Wt of the preputial glands mg	Wt of the preputial glands mg/100 g body wt
I	Hypertensinogen	108	109	30.1	27.9 ± 2.2
II	ACTH	108	104	44.6	43.4 ± 2.3

TABLE II. Effect of 9 Days Treatment with ACTH.

Group	Treatment	Body wt, g Initial	Body wt, g Final	Wt of the preputial glands mg	Wt of the preputial glands mg/100 g body wt
I	Hypertensinogen	110	129	32.4	25.3 ± 1.2
II	ACTH	110	108	44.8	40.8 ± 2.7

preputial glands was due to some pituitary principle other than ACTH(2). Until a short time ago, it was generally assumed that ACTH exerts all its effects only through the adrenal cortex(1). However, recent observations made in this Institute demonstrated that in adrenalectomized rats, maintained on threshold doses of cortisone, ACTH causes pronounced thymic involution. Hence, it was assumed that the hormone must possess some extra-adrenal effect, even though the latter fails to manifest itself in the absence of corticoids(4).

In the present communication we wish to report upon observations concerning an extra-adrenal action of ACTH on the preputial glands. Since the latter are sensitive to sex hormone stimulation, all experiments were performed on animals both adrenalectomized and ovariectomized.

As we shall see, a definite preputial gland stimulation was obtained in these experiments in the absence of both steroid producing glands and of any sensitization by steroids.

Experimental procedure. Two experiments were performed on a total of 40 female piebald rats. In each of these we used 2 groups of 10 rats. The first group received *hypertensinogen* in order to determine the possible effect of non-hormonal foreign protein. This was given at a daily dose level of 6 mg, in the form of 6 subcutaneous injections of 1.0 mg in 0.1 ml of saline, every 4 hours.

ACTH (Connaught Laboratories, Toronto, Lot No. 2-2, 3-1) was given at the same dose level and in the same manner as the hypertensinogen. It will be recalled that in order to obtain maximal adrenal stimulation effects from ACTH, it is essential to give it at short intervals; hence, the injections were made every 4 hours during the day and night.

All the experimental animals, in the 2 series, were bilaterally *adrenalectomized and ovariectomized,* 48 hours before the initiation of the injections. Immediately after this, they were given 1% NaCl, instead of tap water as a drinking fluid. The removal of the gonads and adrenals was necessary in order to eliminate both of the known endogenous sources of hormones.

The 2 experimental series differed only in that injections were given in the first experiment during 6 days, and in the second during 9 days. At the end of each experiment, the animals were killed by exsanguination and their preputial glands removed for weighing, and histological study.

Experimental results. The details of the experimental arrangement, the initial and final body weights as well as the weight of the preputial glands expressed in mg and in mg per 100 g of B.W. (the latter with its standard error) are given in Tables I and II. It will be noted that ACTH increased the weight of the preputial glands. Statistical analysis of this difference showed that the augmentation was significant; the value of "P" in comparing the weights of the preputial glands in mg per 100 g was <0.01 in both experiments. Histologic study of these glands showed bigger and more regular acini in the animals treated

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4. Selye, H., *Nature,* 1951, v168, 149.

with ACTH than in the animals treated with hypertensinogen (see Fig. 1 and 2).

Summary. Experiments on adrenalectomized and ovariectomized rats indicate that ACTH stimulates the growth of the preputial glands, even in the absence of both steroid producing endocrines. It is concluded that ACTH possesses actions which are not mediated through either the adrenals or the gonads and which do not even depend upon the simultaneous presence in the body of adrenal or gonadal hormones. It remains to be seen whether this action is due to the corticotrophic hormone itself or to some contamination in the ACTH preparation used.

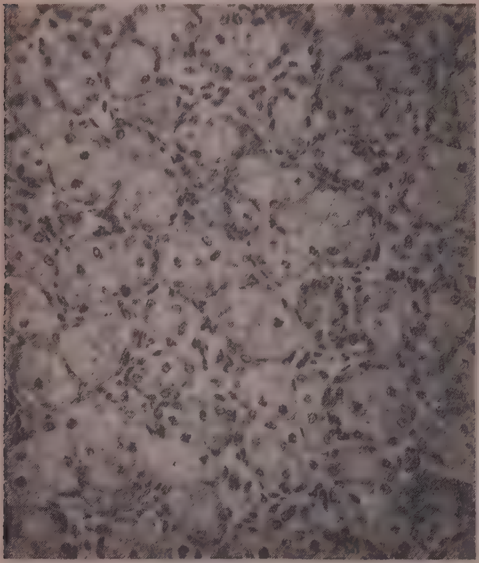

FIG. 1. Preputial gland of a rat treated with hypertensinogen.

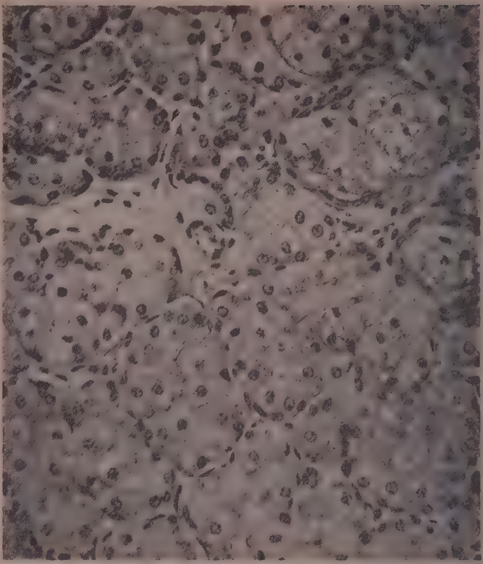

FIG. 2. Preputial gland of a rat treated with ACTH.

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Splanchnic Removal of Bacteria from Blood Stream of Pyrogen-Tolerant Rabbits.* (18968)

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The tolerance produced by the repeated intravenous injection of pyrogenic materials is associated with the development of resistance to the Shwartzman phenomenon(1). Both pyrogen-tolerance and resistance to the Shwartzman phenomenon are blocked by the injection of thorotrast†(2,3). Since the

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* The work was supported by the Anna H. Hanes Memorial Fund and by a grant from the Duke University Research Council.

1. Bennett, I. L., and Cluff, L. E., in mss.

† A stabilized colloidal thorium dioxide prepared by the Heyden Chemical Corp.

2. Beeson, P. B., *J. Exp. Med.*, 1947, v86, 39.

3. Beeson, P. B., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 146.

TABLE I. % Splanchnic Removal of Bacteria in Pyrogen-Treated Rabbits.

Group	No. of rabbits	Pyrogen received	Bacteria infused	% splanchnic removal
Control	10	—	*M. aureus*	62 ± 20 (S.D.)
	11	—	*K. pneumoniae*	20 ± 10 (S.D.)
A	4	Triple typhoid vaccine	*M. aureus*	67 ± 14 (S.D.)
	3		*K. pneumoniae*	8 ± 9 (S.D.)
B	5	PV pyromen	*M. aureus*	68 ± 12 (S.D.)
	4	”	*K. pneumoniae*	15 ± 11 (S.D.)
C	3	P-25 polysaccharide	*M. aureus*	62 ± 19 (S.D.)
	4		*K. pneumoniae*	11 ± 11 (S.D.)

splanchnic removal of bacteria from the blood stream also can be blocked by the injection of thorotrast(4), the present study was undertaken to see whether pyrogen-tolerance in the rabbit is associated with an alteration in the ability to clear the splanchnic blood of organisms.

Methods. Healthy albino rabbits were used. A bacteremia was induced, as in previous studies(5), by the continuous infusion of bacteria into a marginal ear vein. Blood samples were obtained for culture by means of repeated cardiac punctures or catheterization of the inferior vena cava and of the hepatic vein. Anesthesia was maintained throughout each experiment by local metycaine infiltration. The extent of removal of organisms by the splanchnic circulation was calculated subsequently from the simultaneous hepatic venous and either cardiac or vena caval bacterial counts. The organisms used were a hemolytic *Micrococcus aureus* of human origin, coagulase-positive, and an encapsulated *K. pneumoniae type B* (obtained from Dr. J. J. Dubos). A constant infusion of one organism was maintained throughout the first half of the experiment. The infusion was then discontinued, and, after a brief period of no bacterial infusion, the second organism was substituted and infused throughout the latter half of the experiment.

The following pyrogenic materials were used: Triple typhoid vaccine containing one billion typhoid bacilli and 0.75 billion each of paratyphoid A and B organisms per ml (obtained from the North Carolina State Laboratory of Hygiene); P. V. Pyromen, a purified pyrogen from *Proteus vulgaris* (obtained from Dr. L. G. Ginger); and P-25 polysaccharide from *Serratia marcescens* (obtained from Dr. M. J. Shear). *Group A.* Each of 4 rabbits received a daily intravenous injection of triple typhoid vaccine for 10 days on the following schedule: Stock vaccine, diluted 1:6.25 with physiologic saline, 0.25 ml on the 1st day, doubled daily thereafter through the 5th day; stock vaccine, undiluted 1.25 ml on the 6th day, 2.5 ml on the 7th day, 5.0 ml on the 8th and 9th days, 9.0 ml on the 10th day. *Group B.* Each of 4 rabbits received a daily intravenous injection of P.V. Pyromen for 6 days on the following schedule: 100 γ on the 1st, 2nd and 3rd days; 200 γ on the 5th, 6th and 7th days. One additional rabbit received double the above amounts. *Group C.* Each of 3 rabbits received a daily intravenous injection of 0.2 mg of P-25 polysaccharide for 6 days. One additional rabbit received 0.25 mg of P-25 polysaccharide daily for 6 days. In each instance in the above groups, a bacteremia was induced on the day following the last injection of the pyrogenic material.

Results. No significant alteration was observed in the splanchnic removal rate of either *M. aureus* or *K. pneumoniae type B* in the animals which had received pyrogenic material on comparison with the control groups of animals. The data are summarized in Table I.

On inspection of the data, the greatest difference lies between the animals of Group A which were infused with *K. pneumoniae* and the corresponding control group. The difference is not significant statistically ($p > 0.05$).

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4. Martin, S. P., Kerby, G. P., and Holland, B. C., in mss.

5. Martin, S. P., Kerby, G. P., and Holland, B. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 63.

Discussion and conclusions. Beeson(2) has shown that in animals made tolerant to the pyrogenic effect of typhoid vaccine, there is an increased disappearance of the pyrogenic material from the blood stream. The increase in disappearance rate is blocked by the administration of thorotrast. He interpreted this as an indication that the reticulo-endothelial system removes the pyrogenic material from the blood stream and that it does so with increased efficiency on repeated contact with the pyrogen, thus leaving less and less pyrogen in the blood stream to damage other susceptible tissues. More recent studies by Bennett and Cluff(1) have shown that rabbits made tolerant to pyrogens become non-reactive also to the Shwartzman phenomenon. Both of these effects are blocked by the injection of thorotrast(2,3), as is the removal of bacteria from the blood stream(4). However, although there seem to be some similarities between the characteristics of resistance to the Shwartzman phenomenon and resistance to pyrogen effect, the third effect (*i.e.* removal of bacteria from the blood) does not show a cross-relationship, at least insofar as alteration of bacterial removal by pyrogen-tolerance is concerned. The extent of splanchnic removal of bacteria from the blood stream of pyrogen-tolerant animals is not significantly different from the removal rate observed in the control groups.

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Simultaneous Recording of Pulse Contours on Central and Peripheral Arteries with Mechano-Electronic Transducers. (18969)

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With each heart beat the sharp systolic rise in blood pressure expands the elastic walls of the adjacent aorta. This expansion travels rapidly along the walls of the arteries as an elastic wave of decreasing amplitude and can be felt as the pulse.

Regarding the relationship between the pulse wave and other factors in hemodynamics it must be kept in mind that the contour and velocity of any wave are determined by the energy generating it and the elastic qualities of the medium through which it travels. The energy in this case amounts to the product of stroke volume × blood pressure. Hence, these factors determine the pulse wave in principle. The medium transmitting the pulse wave is the wall of the blood filled vessels. The elastic properties of the arterial wall are based upon two components: The elastic fibers which are of passive nature and the muscular elements capable of active contraction. The tone of the muscle fibers in the arterial wall exerts a decisive influence on the velocity and contour of the pulse wave.

An evaluation of the pulse wave therefore offers a means of estimating not only peripheral vascular resistance, but also vasomotor activity as governed by the autonomic nervous system. Despite the importance of these factors in physiological research and particularly in clinical medicine, comparatively few efforts have been made to render the principle more widely applicable.

The principal functions involved are: 1. Systolic and diastolic blood pressure; 2. Diameter of the aorta; 3. Pulse wave velocity measured by simultaneous recording of the pulse contours on at least 2 different sections of the arterial system. The method described here pertains to the last item.

The air transmission sphygmograph as first developed by Frank(1), Wiggers(2), and others has been most frequently used to record pulse contours. This method, although offer-

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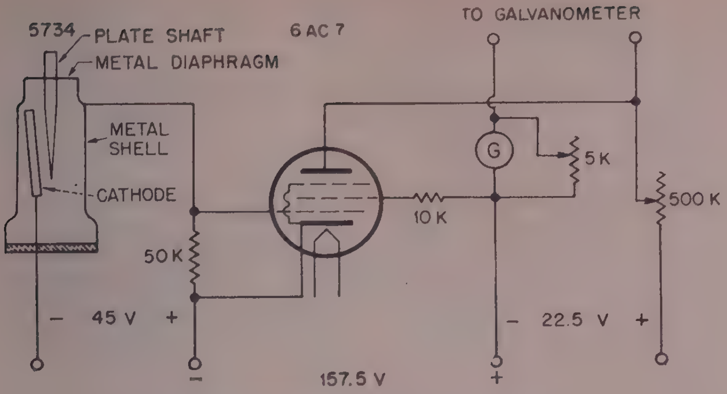
1. Frank, O., *Z. f. Biol.*, 1930, v89, 274.

2. Wiggers, C. J., Modern aspects of the circulation in health and disease, 1923, Lea and Febiger, Philadelphia.



TRANSDUCER TUBE AND AMPLIFIER
FIG. 1.

ing reliable results in the hands of experienced investigators, has certain limitations.

For proper damping and adequate frequency the length of the tubes must not exceed 2 feet(1). This is a disadvantage since movements of the patient or test subject, as on a tilt table, are limited. Furthermore, the sensitivity of the capsules depends upon the air pressure, which makes the system unsuitable for studies at low barometric pressure and at extreme temperatures.

It seemed likely that a decided improvement in the recording system could be effected by transducing the arterial pulsation into changes of an electrical current. The pickup, which would be called a mechano-electric or mechano-electronic transducer, must be highly sensitive, as only very small forces are available. Moreover, the pressure exerted by the pickup on the artery must not exceed the diastolic pressure, in order to avoid distortion of the pulse wave(1). Good results were obtained when piezo-quartz was used as transducer in combination with the recording cathode-ray oscillograph(3). But this device calls for extremely high amplification, and the amplifier must have a long time constant unless D.C. amplification is employed. Furthermore, it is not easy to adjust.

In recent years several reliable types of mechano-electric transducers have become available for biological purposes. In view of the exceedingly small forces operating on such a pickup from the pulse through the skin a mechano-electronic transducer in the shape of a subminiature tube was used in the present investigation. This transducer (RCA-5734), originally designed for application in phonograph pickups and microphones(4) has a natural frequency of 13,000 cycles per second and is specially matched for further D.C. amplification. Superficially the tube resembles a common subminiature radio tube. The difference lies in the shape of the plate. This consists of a conically shaped bar which coincides with the axis of the tube (Fig. 1). The plate shaft, fixed in a small metal diaphragm, protrudes as a small pin out of the tube. The indirectly heated cathode runs parallel to the conical side of the plate shaft. Movements of the projecting pin alter the distance between plate shaft and cathode, thus the inner resistance of the tube is changed in proportion to the deflection. This in turn effects the plate current. Therefore, proportional voltage variations will occur in a resistor on the plate circuit and can be picked up by an amplifier.

In order to transfer the pulse wave motion from the skin to the tube, a suitable adapter was constructed. This consists of a frame which holds the transducer tube and a flat

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3. Tavel, F. V., *Helv. Physiol. Acta*, 1943, v12, Suppl. I, 1.

4. Olson, H. F., *J. Acous. Soc. Am.*, 1947, v19, 307.

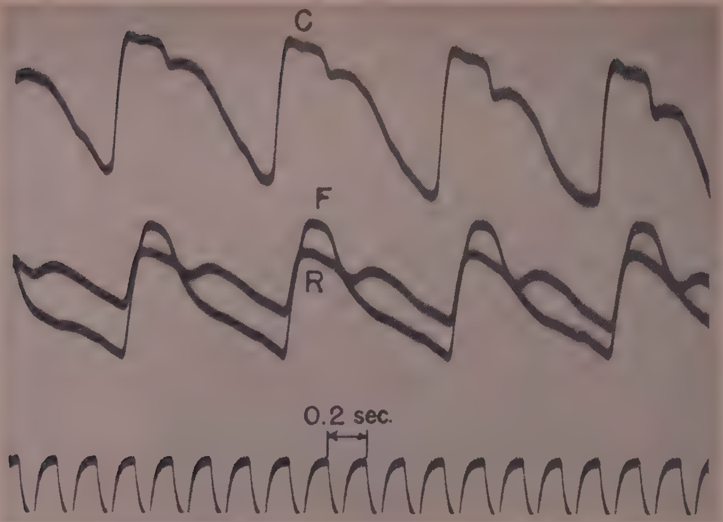

FIG. 2. Optical recordings of pulse curve on carotid (C), radial (R), and femoral (F), arteries.

spring with a small pelotte. Each frame is shaped according to the anatomy of the required location: to the neck for the carotid, to the wrist for the radial, and to the inguinal region for the femoral artery. When the frame is fixed in position with a strap and the pelotte is seen to move over the artery, the pin of the tube is brought into contact with the spring by an adjusting screw. Voltage changes representing the transduced pulse wave are fed into one stage amplifier by direct coupling to the grid circuit, thus avoiding disturbing resonances by using a coupling network. A dial microammeter inserted in the plate circuit, which is equipped with the necessary resistors and is compensated for the basic current, indicates a pulse wave directly. Another galvanometer of the mirror type with an adequate natural frequency records the pulse waves on a photokymograph. The advantages of remote recording are implicit in this arrangement. A combination of 3 complete pickup units has been assembled for simultaneous recording on 3 different arteries. A sample of the recordings obtained is shown in Fig. 2.

From the recordings, the pulse wave velocity can be determined by measuring the time interval between corresponding points on the anacrotic part of the pulse contour on 2 arteries at a measured distance from the heart. The analysis of the pulse contours is carried out in the same manner as in sphygmographic records obtained by the air transmission sphygmograph.

Summary. The application of mechano-electronic transducers is described for the simultaneous recording of arterial pulse contours on central and peripheral arteries in man. The principal advantages of this device over air transmission sphygmographs are remote recording and greater freedom of movement for the subject. The instrument is not affected by changes in environmental temperature or barometric pressure.

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Chronic Epilepsy in the Monkey Following Multiple Intracerebral Injections of Alumina Cream. (18970)

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Previous studies have indicated that subcortical intracerebral injections of small quantities of alumina cream were occasionally effective in producing seizures and electroencephalographic (EEG) abnormalities in the monkey(1,2). A new technic based on simultaneous multiple intracerebral injections of alumina cream is here described.

Method. Eight injections (0.1 ml each) of alumina cream were made into the exposed sensori-motor cortex of one cerebral hemisphere in each of 5 *Macaca mulatta,* and into both hemispheres of an additional animal. Hypodermic needles (gauge 26) were inserted almost perpendicularly to the surface of the cortex in 8 standard positions (Fig. 1) to a depth of 3 to 11 mm and 0.1 ml of alumina cream was injected slowly into each site. An estimation of the amount retained in each hemisphere was noted and found to vary from 0.4 to 0.6 ml. The animals were observed for periods up to 6 months during which time serial EEG's were recorded. The brains were examined post-mortem and the position of the lesions plotted.

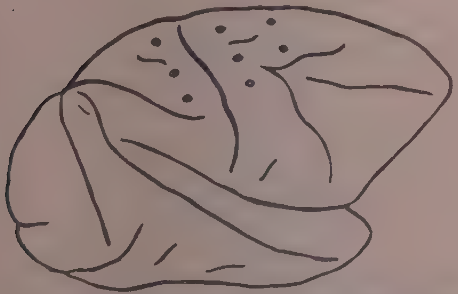

FIG. 1. Diagram of lateral aspect of cerebral hemisphere of *Macaca mulatta* in which the injection sites in the sensori-motor area are indicated by heavy dots.

Results. Five of the 6 monkeys exhibited motor seizures 3-4 weeks following injection. These were usually limited to the contralateral side of the body in 4 of the 5 monkeys injected unilaterally. The convulsive activity was characterized initially by frequent, almost continuous muscle twitching of variable intensity involving the upper and/or lower extremity, and appeared to be accentuated after excitement and movement. Associated loss of consciousness was not observed. In some animals prolonged rhythmic clonic movements of the contralateral extremities became a predominant feature. After a period of several weeks this type of spontaneous motor activity was less evident. Generalized seizures with apparent loss of consciousness occurred infrequently. A moderate degree of paresis was usually present in the limbs of the side contralateral to the injected hemisphere. The monkey injected bilaterally exhibited the most severe reaction: *status epilepticus* for 3 weeks which despite ample luminal sodium treatment terminated in death.

EEG's showed considerable spike, sharp, and slow wave activity which was most prominent over the injected hemisphere. In the later stages metrazol administered intravenously (0.1 ml 10%) accentuated the spike activity and occasionally precipitated associated contralateral clonic movements. When a single dose failed to evoke this response, 3-4 repeated injections proved effective.

Examination of the brain at post-mortem revealed that in all instances an adherent dural scar was present over the entire injected cortical area. Histologically the lesions resembled those described previously(2). The cerebral lesions produced by the injected alumina cream extended from the cortex into the adjacent underlying white matter along the needle track in the "arm-leg" zone. In the animal which failed to develop convulsive attacks a lesion extending into the superior pole of the underlying putamen and adjacent internal capsule was evident.

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* Neurological Division, St. Vincent's Hospital, New York.

1. Kopeloff, N., Kopeloff, L. M., and Pacella, B. L., *Epilepsy,* Edited by Hoch and Knight, Grune & Stratton, New York, 1947, 163.

2. Kopeloff, N., Whittier, J. R., Pacella, B. L., and Kopeloff, L. M., *EEG Clin. Neurophysiol.,* 1950, v2, 163.

Discussion. Our findings indicate that multiple intracerebral injections of alumina cream into one hemisphere caused a more rapidly developing convulsive state than that usually induced by a disc containing alumina cream(1). The motor manifestations were initially constant, focal, variable in intensity, and associated with some weakness of the contralateral limbs. In this respect they resembled the form of motor epilepsy in man described as *epilepsia partialis continua* (Koshewnikow)(3).

The only animal which failed to exhibit convulsive movements after the multiple injection treatment of one cerebral hemisphere had received the deepest injections (11 mm), and the finding of a lesion extending into the superior putamen and internal capsule in this case may be significant. It might be inferred that under these conditions abnormal motor activation from the overlying cortical-subcortical zone was not possible because of interruption at the basal ganglia level.

Summary. 1. Chronic experimental epilepsy was induced in 4 of 5 monkeys (*Macaca mulatta*) by multiple intracerebral injections of alumina cream in the sensori-motor area of one cerebral hemisphere. 2. The early motor convulsive manifestations in the epileptic animals resembled those described in man as *epilepsia partialis continua* (Koshewnikow). 3. In an additional monkey treated by multiple intracerebral injections of alumina cream into both hemispheres death in *status epilepticus* occurred 3 weeks after the onset of seizures. 4. EEG's showed marked spike, sharp, and slow wave activity which was most prominent over the injected hemisphere.

The authors wish to thank Frances Simpson, Elaine Rieders, Frances Zompakos, and Bernice Faigel for their technical assistance.

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3. Koshewnikow, A. J., *Neurol. Centralbl.*, 1895, v14, 47.

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Effect of Hyaluronidase on Electrolytes (Sodium and Potassium) of Dog Synovial Fluid. (18971)

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(Introduced by Emmett B. Carmichael.)

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The action of hyaluronidase as shown by Duran-Reynals(1), is that of depolymerizing a mucopolysaccharide hyaluronic acid, which occurs in high concentration in synovial fluid.

Since hyaluronic acid has been shown to be in a depolymerized state in synovial fluid of arthritic joints(2), and since electrolytes seem to be primarily involved in arthritis(3-5), tests were undertaken to determine the effect of hyaluronidase on the sodium and potassium content of synovial fluid.

Materials and methods. Twenty mongrel dogs were anesthetized by intraperitoneal injection of 100 mg of sodium phenobarbital per

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1. Duran-Reynals, Francisco, *Comp. rend. soc. biol.*, 1928, v99, 6.

2. Ragan, Charles, and Meyer, Karl, *J. Clin. Invest.*, 1949, v28, 56.

3. Jackson, M. D., *Am. J. Med. Sc.*, 1942, v204, 540.

4. Selye, Hans, *Canad. Med. Assn. J.*, 1944, v50, 426.

5. Selye, Hans, *British Med. J.*, 1949, v2, 4637.

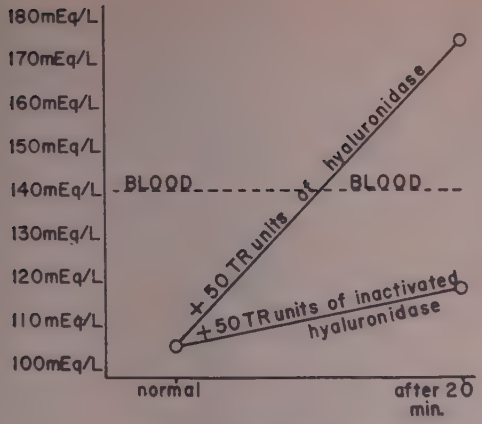

FIG. 1. Effect of hyaluronidase on sodium of synovial fluid.

kilo of body weight. Normal synovial fluid was aspirated from identical joints of both hind legs of each dog in amounts as near to 0.1 cc as possible. The needles were left in place and into one joint of each dog was injected 50 turbidity reducing units of purified bovine testicular hyaluronidase* dissolved in 0.1 cc of distilled water. As a control, the same amount of hyaluronidase which had been inactivated by heating at a temperature of 75°C, was injected into the other joint. After a 20-minute period both knee joints were again aspirated, and the amounts of fluid obtained were similar to those of the previous aspirations. Blood was then obtained by arterial puncture for serum analyses. Sodium and potassium determinations were performed on all specimens by means of the Model DU Beckman Flame Spectrophotometer, using modifications of the Mosher technic(6).

Results. The concentrations of sodium are expressed graphically in Fig. 1. Normal levels of sodium were found to have a mean value of 103.8 mEq/L. Seventy per cent of the samples were within one standard deviation from the mean. After the action of hyaluronidase the mean concentration was 174.6 mEq/L, with 69.2% of the samples falling within one standard deviation from the mean. The difference between these 2 means was 70.8 mEq/L, or 9.1 standard errors. Identical controls run with inactivated hyaluronidase showed a change of only 12.2 mEq/L. Seventy-five per cent of the samples were within one standard deviation from the mean.

The potassium concentrations are expressed graphically in Fig. 2. The values for potassium in normal synovial fluid were found to have a mean of 3.2 mEq/L. Sixty per cent of the samples were within one standard deviation from the mean. Hyaluronidase increased the mean concentration to 7.6 mEq/L, with 50% of the samples being within one standard deviation from the mean. The result was a difference of 4.4 mEq/L, or 4 standard errors between the means. The controls showed a slight decrease of 0.3 mEq/L, probably due to dilution of the normal fluid.

Sodium and potassium concentrations of blood serum were respectively 139.8 mEq/L and 4.3 mEq/L.

It was observed that the viscosity after injection of hyaluronidase was markedly decreased. Because of this difference, the samples were diluted sufficiently so that the accuracy of the instrument would not be affected.

Discussion. It has been shown both *in vivo* and *in vitro* that hyaluronidase depolymerizes the hyaluronic acid of synovial fluid(7), and

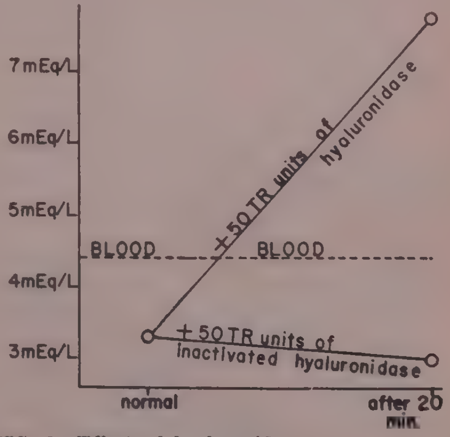

FIG. 2. Effect of hyaluronidase on potassium of synovial fluid.

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* The hyaluronidase (Wydase) used in this was kindly supplied by Wyeth, Inc., Philadelphia, Pa.

6. Mosher, R. E., *Am. J. Clin. Path.*, 1949, v19, 461.

7. Ragan, Charles, and De Lamatar, Arlene, PROC. SOC. EXP. BIOL. AND MED., 1943, v50, 349.

further that the permeability of the synovial membrane is altered by its action(8). Ragan has demonstrated that the synovial fluid of arthritic joints occurs in a depolymerized state(2). There has been some controversy as to the cause of this depolymerization, but Bauer(9) as early as 1940 observed the similarity between arthritic joint fluid and "mucin" after the action of "mucinase."

The importance of electrolyte changes in arthritis has been suggested by experimental evidence. The administration of NaCl solution to rats followed by desoxycorticosterone (DOCA) administration resulted in rheumatic lesions in the heart(4). Selye(5) has produced arthritis in rats by DOCA intoxication, and has further shown that DOCA aggravates formalin induced arthritis. It is interesting to note that DOCA has no effect on the action of hyaluronidase(8). Cortisone, along with certain other compounds used in the treatment of arthritis, inhibits hyaluronidase(8,10).

In view of all this evidence it seemed of interest to study the relationship of the electrolytes of the synovial field to the action of hyaluronidase. The experiments performed showed that hyaluronidase, in addition to decreasing the viscosity of synovial fluid, produced a significant increase in the Na and K levels. Since the resultant concentration was significantly greater than that of the blood it may be assumed that the increase is not due to trauma. Furthermore, the controls failed to show any increase. Analyzing the data statistically it was found that the difference between the means was highly significant.

Conclusions. (1) The action of hyaluronidase increases the concentrations of both sodium and potassium in the synovial fluid of the dog. (2) Inactivated hyaluronidase has no action on the sodium and potassium in dog synovial fluid.

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8. Seifter, J., Beader, D. H., and Bagany, A. J., Proc. Soc. Exp. Biol. and Med., 1949, v72, 277.
9. Bauer, W., Ropes, Marion W., and Waine, Hans, *Physiol. Rev.*, 1940, v20, 272.
10. Jaworski, A. A., Farley, John E., Barrett, John, and Jaworski, Rudolf, *A. J. Ped.*, 1950, v37, 597.

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Distribution of Esterase in the Mouse Embryo.* (18972)

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The distribution of esterase activity in adult mammalian tissues has been investigated by Gomori and others(1-5). The present study extends these observations to embryonic tissue.

Materials and methods. Strain A mouse embryos measuring 5, 10, 15, and 20 mm in a crown-rump length were used. Serial sections were made of acetone-fixed embryos for each stage. Adjacent sections were stained with hematoxylin and eosin, and with the Gomori esterase technic(4,6), employing the usual controls.

Results. No esterase activity was demonstrated in the 5 and 10 mm embryos. Activity was shown in both the 15 and 20 mm stages, the reaction being somewhat more intense in the larger embryos. Esterase activity was found in the pancreas, liver, lungs and thyroid, but not in the kidneys. In the pan-

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* Aided by a grant from the National Cancer Institute, U. S. Public Health Service.

1. Gomori, G., Proc. Soc. Exp. Biol. and Med., 1945, v58, 362.
2. ———, *Ibid.*, 1948, v67, 4.
3. ———, *Ibid.*, 1949, v72, 697.
4. ———, *Arch. Path.*, 1946, v41, 121.
5. Montagna, W., and Noback, C. R., *Am. J. Anat.*, 1948, v83, 409.
6. Stowell, R. E., and Lee, C. S., *Arch. Path.*, 1950, v50, 519.

creas the acinar cells gave a strong reaction. In the liver the reaction appeared to be restricted to the parenchymal cells, and was more intense in cells adjacent to the central veins. The kidney gave no reaction. In the lungs the pseudostratified columnar epithelium lining the bronchi showed intense activity. In addition, other cells in the lung appeared to give a slight reaction; these may be the septal cells, since Gomori(4) states that septal cells of the adult lung contain esterase. The acinar cells of the thyroid gland gave a marked reaction both in the 15 and 20 mm stages of the mouse embryo and in the adult. Also active in the embryo were the pseudostratified columnar epithelium lining the nasal cavities and the so-called hibernating glands.

Discussion. The occurrence of esterase activity in the pancreas, liver, hibernating glands, and bronchial epithelium is in agreement with previous observations in adult mice and other mammals(4). Esterase activity has not previously been reported in the nasal mucosa; however, this activity might be expected, since the closely related bronchial epithelium contains the enzyme.

The esterase activity of both the embryonic and adult thyroid gland of the mouse is of particular interest since esterase has not been demonstrated in the thyroid glands of other animals previously studied. For example, Gomori(4) reports the absence of a histochemical reaction for esterase in the thyroid glands of man, monkey, dog, rabbit, guinea pig, and rat. Furthermore, Huggins and Moulton(7) who have made chemical studies of esterase activity in various organs of the rat and dog, report that the thyroid glands of these forms do not show a high level of esterase activity.

Quantitative microchemical methods are now being applied to embryonic organs to determine more precisely the rate of increase of esterase activity during development. In addition, because of the greater sensitivity of the chemical methods, esterase activity may be traced in the earlier embryonic stages which do not contain enough enzyme to give a positive histochemical test.

Summary. The 5 and 10 mm stages of mouse embryo do not show any histochemical evidence of esterase activity by the technic of Gomori. The distribution of esterase in the 15 and 20 mm stages of the mouse embryo, as demonstrated by the histochemical technic of Gomori, is essentially the same as that in the adult mouse. The only exception is the kidney, which does not give a positive staining reaction in the embryo. The occurrence of esterase activity in the thyroid gland seems to be peculiar to the mouse; the significance of this observation is not understood.

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7. Huggins, C., and Moulton, S. H., *J. Exp. Med.*, 1948, v88, 169.

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Extension of Motile Life Span of Spermatozoa of the Domestic Fowl by Amino Acids and Proteins. (18973)

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Recent experiments(1,2) with sea urchins and other marine animals have shown that the addition of any one of a number of different amino acids and peptides to suspensions of the spermatozoa in sea water prolongs very greatly their functional life span. Both the

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* Division of Poultry Husbandry, University of California, Davis. The experimental work was done at the Kerckhoff Laboratories of Biology while the senior author was on sabbatical leave at the California Institute of Technology.

1. Tyler, A., *Biol. Bull.*, 1950, v99, 324.

2. Tyler, A., and Atkinson, E., *Science*, 1950, v112, 783.

TABLE I. Influence of Glycine on Duration of Motility of Cock Sperm.

				Max motility after 1st hr		Duration of motility		
Glycine conc., molar	Control sol'n	No. of exp.	Semen conc., %	Control, %	Exp., %	I control, hr	II exp., hr.	Relative life span (II/I)
.033	⅓ s.w.	4	.3– .5	0	75– 90	<.1	6.5	>65
.033		4	.6– .7	0–75	75–100	<.1–4.5	>10.2–>11.1	2.3–>100
.033		3	1 –1.6	10–100	100	2.5–5.9	7–>11	1.4–>4.4
.067–.133		6	.6	10–75	25–100	2 –3	7.5–>11.1	2.5–>5.6
.167		2	.6	10–75	1	2;3	3.5;2.5	1.8;0.8
.013	Saline #1	1	.4	<1	5	1	8	8
.03		4	.3–.4	<1–1	25–100	1–3.5	8–10	2.9–10
.03		4	.6–1	1–10	50–100	2–3.5	7–>13	3.5–>4.5
.03	Saline #2	3	.3	10–75	50–100	4–7	8.5–13	1.8–2.1
.03		3	1 –1.2	25–50	75–90	7–14	9–>31	.6–2.4
.03		3	2.9–5	50–75	75–90	6.7–28	13.5–38	1.4–2

duration of motility and of fertilizing capacity are prolonged in presence of these agents. The present paper deals with the results of tests of these agents on cock sperm.

Material and methods. Semen was obtained by the method of abdominal massage(3) from single comb White Leghorn and Black Minorca cocks.[†] The semen samples were collected in glass tubes which were then placed in a thermos jug containing water at 15°C. The tubes were held above the water level so as to avoid too rapid cooling and possible temperature shock. The samples were kept in this condition for ½ to ¾ of an hour before dilution in the test solutions. The diluted sperm were incubated in a water bath at 22°C, using 10 ml samples in 50 ml glass stoppered Erlenmeyer flasks. In most experiments the flasks were shaken at 80 c.p.m. and 5 cm excursion. The percentage of motile sperm was estimated at regular intervals, microscopically, using semi-dark-field illumination. The total duration of motility or "life span" of the suspension is reported as the closest estimate of the time at which all the spermatozoa had become motionless.

A total of 53 tests were made with glycine at concentrations ranging from 0.013 to 0.167 M in salt solutions and with sperm densities ranging from 0.2 to 25% of semen. Two types of control diluents were used in different experiments: (a) sea water diluted 3-fold with distilled water to bring the osmotic pressure to a value tolerated by avian spermatozoa, and (b) 0.85% NaCl containing 0.214% $NaHCO_3$ and adjusted to pH 8.0 (saline No. 1). Ordinary distilled water and reagent-grade NaCl was used for saline No. 1. In certain later experiments glass redistilled water and Merck's NaCl for biological purposes was used for the preparation of the solution, and this is designated as saline No. 2. In the individual experiments the glycine solutions were prepared with the corresponding control diluent, using a stock glycine solution isotonic with the salt solution employed and at pH 8.0.

Results In Table I the results of 37 separate tests with glycine are summarized. That glycine may prolong the life span of cock spermatozoa is evident. In all of the experiments, with two exceptions to be discussed below, motility persisted longer in the solutions containing glycine than in the control solutions. The relative prolongation varied from 1.4 to more than 100 fold. A high degree of motility (*i.e.*, a high percentage of motile sperm) was also maintained better in these glycine solutions. This is illustrated in Table I by the maximum percentage motility observed after the first hour. There is, however, an upper limit to the effective concentration of glycine as demonstrated by the results

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3. Burrows, W. H., and Quinn, J. P., *Poultry Science*, 1937, v16, 19.

[†] The animals used were from a flock at the George A. Richardson Breeding Ranch in San Gabriel, Calif. We are indebted to Mr. Marshall Richardson for placing these animals at our disposal and for his generous cooperation.

TABLE II. Effect of Concentrations of Glycine and of Semen on Duration of Motility of Cock Sperm. Experiment with a single pooled semen sample in Saline #2.

Glycine conc., molar	Semen conc., %	Max motility after 1st hr, %	Duration of motility, hr	Relative life span
0	.02	1	1	—
.03		5	7	7
.065		25	8	8
.1		50	6	6
.15		50	4	4
0	1	10	6	—
.03		10	8	1.3
.065		50	16	2.7
.1		75	8	1.3
.15		75	6	1
0	5	50	16	—
.03		90	24	1.5
.065		90	9	.6
.1		100	9	.6
.15		75	5	.3
0	25	90	32	—
.03		90	40	1.2
.065		90	50	1.6
.1		100	48	1.5
.15		90	50	1.6

with this amino acid at 0.167 M concentration. Glycine is isotonic with the control solution at this concentration and, consequently, was used without addition of any saline. In this solution life span was not prolonged and the degree of motility decreased rapidly, suggesting that either the high concentration or lack of salts may be toxic to the sperm. Significant prolongation was obtained with solutions ranging from 0.013 to 0.133 M (Table I). In an additional experiment, in which the total life span was not determined, sperm in solutions containing as little as 0.0033 M glycine were 50 to 75% active at least an hour after all sperm in the control solutions were dead.

As is well known, the life span of spermatozoa decreases with increasing dilution of the semen(4). This is evident in the present data on the control suspensions. The extent to which glycine prolongs the life span increases with decreasing concentration of semen, but not enough to overcome completely the effect of dilution (Table I). This tendency is comparable to that reported previously(1,2) for spermatozoa of invertebrate species.

Tyler and Rothschild(5) suggested that one way in which the amino acids might act in prolonging the life span of sperm is by binding certain highly toxic heavy metals ordinarily present in trace amounts in the diluents. On the basis of this consideration we prepared the saline No. 2, which contains considerably less of the heavy metals than the saline No. 1. In two direct comparisons of salines No. 1 and No. 2 using 0.3% semen, the life span was 1.2 hours in saline No. 1 and 4 and 5.5 hours in saline No. 2. With higher semen concentrations the life span in saline No. 2 was even more prolonged, and the relative prolongation due to glycine was correspondingly reduced. Glycine was still effective, however. In all but one of the trials listed in Table I the sperm lived longer in glycine than in saline No. 2.

A comparison of the relative effectiveness of semen concentration and glycine concentration on life span in saline No. 2 was obtained in a single experiment using one pooled sample of semen (Table II). The effect of semen concentration is clearly shown. Glycine appears to be only slightly effective in the higher sperm concentrations, but very considerably so in 0.2% semen. The optimum glycine concentration was 0.065 M, but, as in the results summarized in Table I, concentration was not sharply critical.

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4. Rothschild, Lord, *Biol. Rev.*, 1951, v26, 1.

5. Tyler, A., and Rothschild, Lord, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 52.

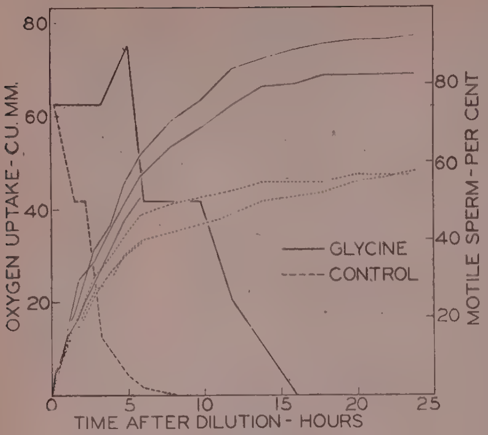

FIG. 1. Cumulative oxygen uptake and motility of senescing cock spermatozoa (5% semen suspension) in saline #2 with and without glycine (0.03 molar); (heavy lines denote sperm motility, light lines O_2 uptake).

Preliminary tests were also made of the effect of glycine on the oxygen uptake of cock sperm. The measurements were made in triplicate on 5% semen in conical Warburg flasks with saline No. 2 and with 0.03 M glycine in saline No. 2, each containing 50 units per ml of penicillin G. The antibiotic has proved useful(5) in inhibiting bacterial growth during prolonged respiration measurements on sperm suspensions. The degree of motility was followed on parallel samples in ordinary flasks and occasional samples from the respiration flasks. In this experiment the extension of motile life in the presence of glycine (Fig. 1) was much greater than that obtained in previous experiments (Table II) at this semen concentration. The total oxygen uptake in the presence of glycine considerably exceeds that of the controls, correlating roughly with the increased duration of motility. As in the experiments with sea urchin sperm(5) the presence of the amino acid enables these cells to maintain their oxidative metabolism longer than in its absence. Whether or not there are also significant initial differences in rate cannot be determined from the present measurements.

Diluted egg yolk and egg white are also effective in prolonging motility; 1% of the former and 2.5% of the latter in saline No. 1 produced prolongations approximately equivalent to optimum concentrations of glycine. Consequently, the effects of individual egg white proteins were studied. Of especial interest, in view of the hypothesis stated above, was the relative effectiveness of conalbumin, which binds iron and copper strongly(6,7), and the other components of egg white. For the same reason a derivative of an egg white protein, hydroxylamidoovomucoid, which is also a strong iron binder(6) was included in this study.[‡] In saline No. 1 conalbumin prolonged the life span of sperm nearly twice as much as ovomucoid or as a preparation of egg white globulins (Table III). In saline No. 2 the relative effectiveness of these materials was the same, but the effects were only about half as great. Ovalbumin, which was studied only in saline No. 2, had no effect, but hydroxylamidoovomucoid more than doubled the life span in saline No. 2. In an additional experiment using saline No. 2 and 5% semen (not recorded in Table III) none of the proteins were effective, with the possible exception of hydroxylamidoovomucoid, which gave a relative life span of 1.3.

Discussion. Relatively few studies have been made of the life span of avian sperm *in vitro*. Grodzinski and Marchlewski(8) examined the effect of degree of dilution of the semen, temperature, pH, and certain diluents. The effectiveness of the diluents in maintaining motility was found to be, in decreasing order, avian blood serum, embryonic extract, egg white, and Tyrode's fluid. Later (9) they found that the avian sperm would survive in the blood serum of various vertebrates, but the results were complicated by the presence, in many of the sera, of natural

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6. Fraenkel-Conrat, H., *Arch. Biochem.*, 1950, v28, 452.

7. Fraenkel-Conrat, H., and Feeney, R. E., *Arch. Biochem.*, 1950, v29, 101.

[‡] The egg white proteins were prepared at the Western Regional Research Laboratory, Albany, Calif., and kindly supplied through the courtesy of Dr. R. E. Feeney.

8. Grodzinski, Z., and Marchlewski, J., *Bull. Acad. Polonaise des Sciences et des Lettres,* Cracovie, (Class des Sci. Math. et Naturelles), 1935, Series B, 347.

TABLE III. Influence of Proteins on Duration of Motility of Cock Sperm.

Material	Conc., %	Control sol'n	No. of exp.	Semen conc., %	Max motility after 1st hr		Duration of motility		Relative life span (II/I)
					Control, %	Exp., %	I control, hr	II exp. hr	
Conalbumen	.25	Saline 1	3	.4–.7	<1–10	25–100	1–3.5	9–11	3.1–10
	.1	1	1	.4	<1	10	1	6	6
	.25	2	1	1	25	25	7	26	3.7
Ovomucoid	.25	1	3	.4–.7	<1–10	1–50	1–3.5	3–4.5	1.3–3
	.1	1	1	.4	<1	<1	1	3.5	3.5
	.25	2	1	1	25	75	7	9.5	1.4
Globulins	.25	1	3	.4–.7	<1–10	5–10	1–3.5	4–6	1.7–4
	.1	1	1	.4	<1	1	1	4	4
	.25	2	1	1	25	25	7	12	1.7
Ovalbumin	.25	2	4	.3–2.9	25–75	25–90	7–<13	7–<13	.9–1.2
Hydroxylamido-ovomucoid	.25	2	3	.3–1.2	25–75	25–95	7–13	12–24	<1–3.4

heteroagglutinins for avian sperm. Various studies(10-13) have been made of the effect of storage temperature on fertilizing capacity of undiluted sperm, and the optimum is reported(13) to be between 10° and 20°C.

Munro(14) examined immediate effects of temperature and various diluents on motility without attempting to study its duration. He found that the motility of fowl sperm was lost at body temperature when suspended in physiological saline, Ringer's, Baker's, or Milanov's solution and in fluids expressed from the infundibulum and magnum, but he observed a high degree of motility in these diluents at lower temperature. Distilled water, seminal fluid, blood serum, uterine fluid, and egg white, on the other hand, supported motility at all temperatures studied (75-105°F). He(15) observed that the fertilizing capacity of sperm used immediately after dilution was reduced much more in Milanov's solution than in seminal fluid. By contrast with the above, Bonnier and Trulsson(16) observed no reduction in fertilizing capacity immediately after dilution with a modified Ringer's solution so long as the final semen concentration was at least 10%.

The results of the present investigation show that motile life span can be greatly extended simply by the addition of an amino acid to the suspending fluid as well as by addition of certain proteins. Whether or not fertilizing capacity is correspondingly extended, as in the experiments with sperm of invertebrates(1,2), has not as yet been determined. The present findings are also consistent with the suggestion(5) that the amino acids and proteins may act by virtue of their ability to bind certain heavy metals which are present in the usual salt solution diluents in trace amounts, and which may be highly toxic to the spermatozoa. Further studies of this and other possible interpretations of the limited duration of life span and fertilizing capacity of diluted sperm are contemplated.

Summary. Glycine, when added to saline diluents at concentrations between 0.003 and 0.133 molar, extends the life span of spermatozoa of the domestic fowl as measured by persistence of motility at 22°C. Glycine was relatively more effective on dilute than on concentrated semen suspensions. Certain

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9. Grodzinski, Z., and Marchlewski, J., *Bull. Acad. Polonaise des Sciences et des Lettres*, Cracovie, (Class des Sci. Math. et Naturelles), 1938, Series B, 55.

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11. Warren, D. C., and Gish, C. L., *Poultry Sci.*, 1943, v22, 108.

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egg-white proteins had a similar effect on the life span of sperm. Conalbumin and a derived protein, hydroxylamidoovomucoid, were more effective than other proteins from egg white. Purification of the saline diluent alone served to prolong the life span of spermatozoa and correspondingly reduce the effects of glycine and proteins.

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Production of Cirrhosis and Liver Tumors in Male Rats Using High Dosages of 2-Acetylaminofluorene.*† (18974)

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Since Wilson, De Eds, and Cox, Jr.(1) introduced 2-acetylaminofluorene (2-AAF) as a new experimental carcinogen, the compound has been widely used, due to its powerful carcinogenic action and the unusual variety of benign and malignant tumors produced in different organs. 2-AAF appears to be of particular value in studies of the mode of development of cirrhosis and liver tumors. The possibility of correlation of liver changes with lesions produced in other locations seems to confer on 2-AAF an advantage over compounds with strictly hepatotoxic action.

Methods. One hundred and forty-eight rats of hooded strain were used, comprising 73 males and 75 females. The rats were derived from a local strain, inbred since 1933 by continuous brother and sister mating. Two animals of the same sex were kept in each cage. The diet was fully adequate, consisting of a standard Purina meal preparation and water *ad libitum.* The 2-AAF was added to the diet (by mixing) for 35 consecutive weeks. The age of the animals at the start of the experiment was 4 weeks in 128 rats (53 males and 75 females) (Group I), and 16 weeks in 20 males (Group II). The absolute dose of the 2-AAF was 4 mg per day in Group I and 7.5-9 mg per day in Group II for the first 7 weeks; afterwards it was lowered to 3 mg and 6 mg per day respectively for the remaining period of time.

It soon became apparent that male rats receiving higher absolute daily dosages of 2-AAF (Group II) developed extensive liver changes in a short period of time. Twelve of them died within 39 days of the start of administration of the compound. Partial hepatectomies were performed at intervals of 4 to 5 weeks for 7 months on the remaining rats of this group, starting from the third month. Under light ether anesthesia and using sterile technic, 20-25% of the liver volume was removed each time through a mid-line abdominal incision. Although the total amount of liver tissue resected by repeated hepatectomies exceeded the original liver volume, the majority of the animals survived all operations. Control hepatectomies were done on 6 animals kept on the lower absolute daily dosage of 2-AAF (Group I). Hematoxylin-and-eosin and connective-tissue-stain sections were prepared from the resected portions of the liver.

Results. Pathological findings observed by repeated hepatectomies. The earliest gross liver changes in the older group of male rats were observed during the second month by examining the 11 rats which died in this period. The liver was decreased in size with multiple white nodules protruding on the surface. Microscopically, the nodules consisted of circumscribed areas of hyperplasia without specific relation to lobular pattern (Fig. 1).

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* This work was supported by a grant-in-aid from the National Cancer Institute of Canada.

† This work was presented at Clinical Congress of American College of Surgeons, Boston, Mass., Oct. 1950. Abstract in *Trans. Surg. Forum,* 1950, v1, 205.

‡ Research Fellow in Experimental Surgery, National Cancer Institute of Canada.

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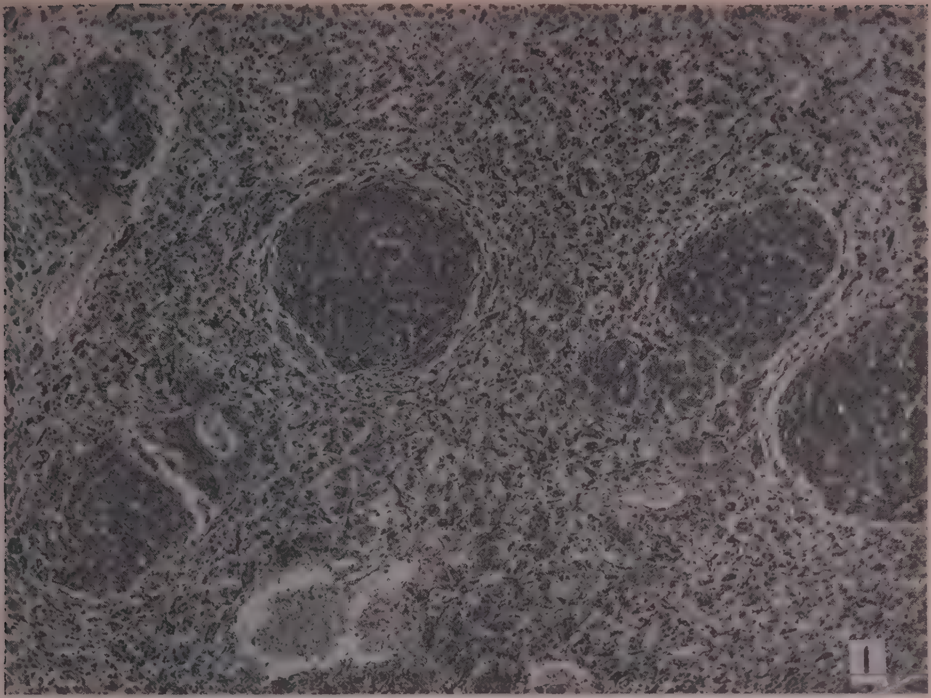

FIG. 1. Irregularly disposed nodules of hyperplastic liver cells, displacing necrotic parenchyma by expansion. H & E. × 105.

The remaining liver tissue showed subacute necrosis, the cells showing disintegrated cytoplasm and degenerative nuclear changes. The general appearance suggested that the degenerated liver tissue was being progressively displaced by multiple nodules of regenerating hyperplastic cells, distorting the normal lobular architecture. In the third month, tissue removed from 8 rats by partial hepatectomies showed complete destruction of the normal lobular pattern (Fig. 2). The first impression, from routine hematoxylin-and-eosin sections, was that of an early cirrhosis, with nodules of regenerating liver cells separated by areas of degenerated tissue. However, connective-tissue stains showed only accentuation of the reticular framework and no definite increase in connective tissue. In the fourth month, active fibroblastic proliferation dominated the histological picture. This, as shown best by Masson and Van Gieson stains, was restricted to spaces between hyperplastic nodules. The cells in the areas of hyperplastic regeneration showed different grades of fatty and glycogenic infiltration. Included in the fibrotic granulation tissue were numerous bile ducts, some of them showing cystic dilatation with flattening of the duct epithelium (Fig. 3). In the fifth month, further increase in the size of the liver was noted in spite of previous resections. Microscopically, this was seen to be due to further increase in size of the hyperplastic nodules. The amount of collagen was denser, and numerous cystically dilated bile ducts were present. In the sixth month, further increase in the collagen was prominent, and associated decrease in cellularity of the fibrotic tissue gave the cirrhosis a more quiescent appearance (Fig. 4). Dilatation of bile ducts had progressed in many cases to cyst formation. In the seventh month, marked increase in hyperplasia was observed. The presence of intermingled fields of normal and hyperplastic cells produced complex "geographic" patterns. In the eighth month, only slight fibrosis was still

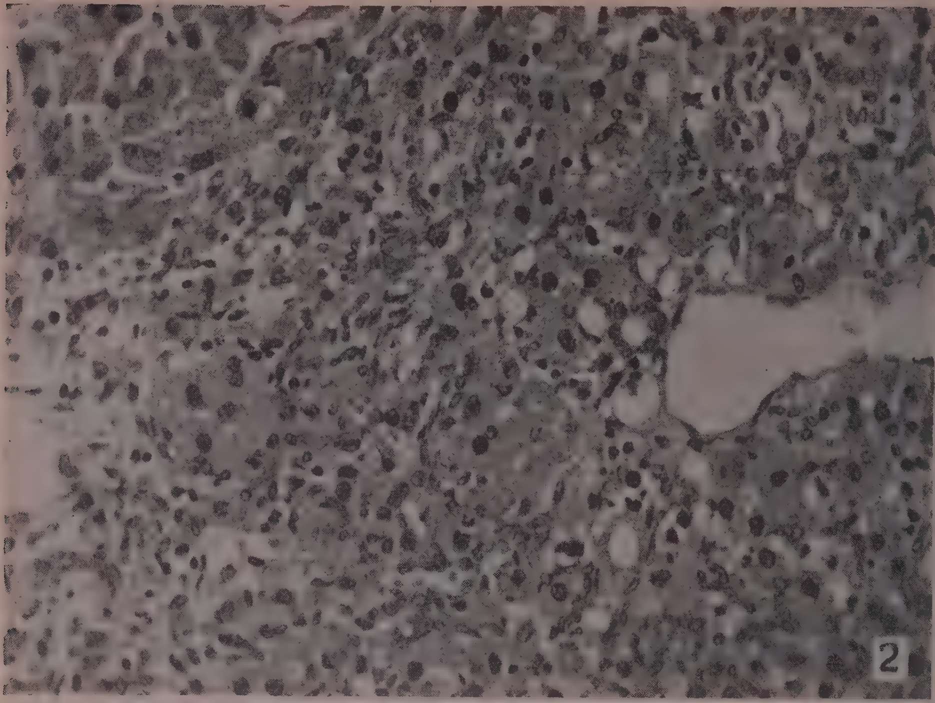

FIG. 2. Beginning fibroblastic proliferation in the necrotic liver parenchyma. Regeneration from intact remaining parenchymal cell groups is evident. H & E. × 415.

present, with narrow, irregular bands of collagen dividing large areas of regenerating parenchyma. In several rats, large areas of proliferated and dilated bile ducts were observed, classified as benign bile-duct adenomas. Liver-cell tumors were present in the majority (Fig. 5).

In the control group of rats, which were maintained on a lower absolute daily dosage of 2-AAF (Group I), the degenerative liver changes were much less extensive. In male rats, the nodules of hyperplastic liver tissue of similar appearance as those described previously, were not as diffuse; the amount of fibroblastic proliferation, as well as parenchymal hyperplasia, was less pronounced. In females of this group, mild degenerative changes appeared considerably later (fifth to seventh months).

Tumors produced by 2-acetylaminofluorene. The average time of occurrence of malignant tumors produced by 2-AAF was between the ninth and twelfth months after the beginning of the experiment. Some of the animals still alive at the end of the twelfth month showed visible or palpable tumors, not included in the results reported here. Ninety rats died after the sixth month, and 35, including 6 males and 29 females, were living at the end of the twelfth month. At autopsies, tumors were found in 70 out of 90 rats; in 43 animals the tumors were classified as malignant on the basis of invasive growth and/or distant metastases. In many cases, multiple primary tumors were present. The most frequent sites of origin were: external auditory canal region (about equal sex incidence), liver (more frequent in males), and breast (only in females). Almost without exception the tumors were of epithelial origin. Out of 31 liver tumors observed, 27 occurred in males and 4 in females. The most frequently appearing tumors were of the liver-cell type with solid, trabecular, or glandular arrangement, often multicentric in origin. Malignant tumors were observed during and after the ninth month, although foci of cells, which might be interpreted as showing incipient malignancy, were

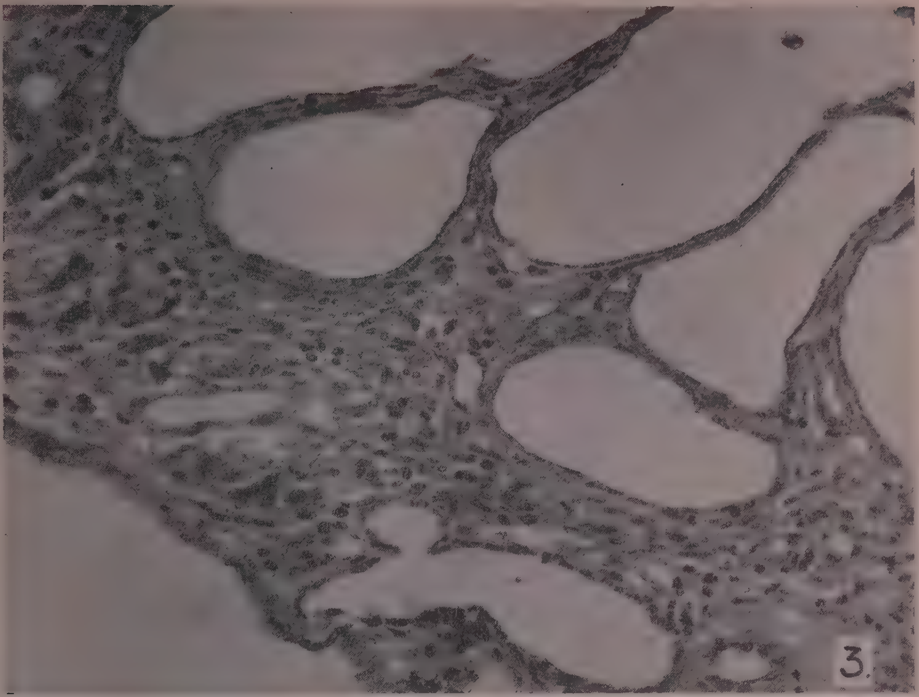

FIG. 3. Cystic dilatation of the bile ducts with fibrosis of the stroma (cholangiofibrosis). H & E. × 440.

observed in the seventh and eighth months. Frequent peritoneal and pulmonary metastases were found. Multiple cysts, sometimes reaching huge sizes, were often present on the tumor and liver surfaces, occasionally giving rise to intra-abdominal hemorrhage. No malignant tumors of bile-duct origin were observed.

Discussion. Studies by repeated partial hepatectomies indicate a sequence of changes in liver structure similar to the "compensatory process following destruction of liver tissue" described by Opie(2) upon administration of azo dyes to rats. The initial lesion observed during the second month of administration of 2-AAF might be summarized as subacute necrosis with nodular hyperplasia. As suggested by the microscopic appearance of the lesion (Fig. 1), the hyperplastic regenerative nodules seem to exert pressure on the surrounding degenerated liver parenchyma by means of expansive growth. This might be considered as the primary cause of accumulation of pre-existing reticular framework fibers in the degenerated liver tissue. These findings, and the fact that nodular hyperplasia precedes fibroblastic proliferation, seem to substantiate the views of Kelty, Baggenstoss, and Butt(3), that the regenerative phase plays a determining role in the production of cirrhosis.

The rapidly progressing hyperplasia of liver cells occurring in later months of administration of 2-AAF caused a relative decrease in the amount of cirrhosis. The newly formed parenchyma seems to develop resistance to the toxic action of the compound when its administration is continued. Thus the type of cirrhosis produced by 2-AAF does not comply with the definition of liver cirrhosis by Kretz(4) as "a chronic degenerative process with inclusion of regenerative periods." Simi-

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2. Opie, E. L., *J. Exp. Med.*, 1944, v80, 231.

3. Kelty, R. H., Baggenstoss, A. H., and Butt, H. R., *Proc. Mayo Clin.*, 1950, v25, 18.

4. Kretz, R., *Wien. klin. Woch.*, 1900, v12, 272.

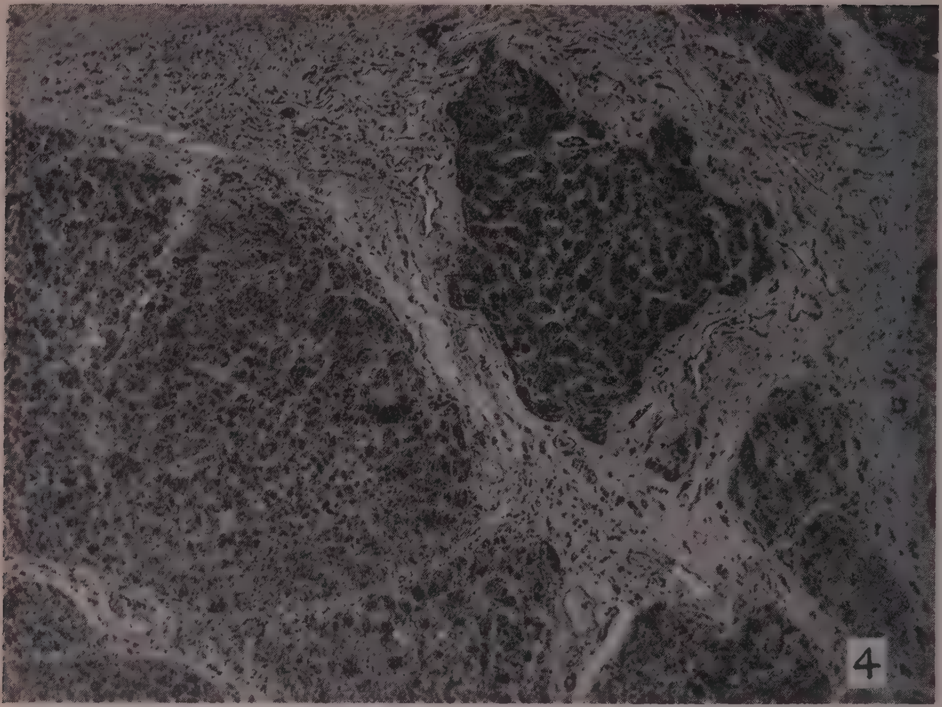

FIG. 4. Advanced cirrhosis with coarse bands of dense collagen containing numerous bile ducts, many of which show ectasia. Masson trichrome. × 110.

larly, Orr(5) expressed doubt that irreversible cirrhosis could be produced by the administration of p-dimethylaminoazobenzene.

Orr(5) drew attention to the fact that cirrhosis produced by certain agents, such as carbon tetrachloride, reaches a much greater density than that produced by p-dimethylaminoazobenzene, although the latter has more potential carcinogenic effect. The same can be stated with regard to the action of 2-AAF. Thus the frequency of liver tumor occurrence cannot be regarded as paralleling the extent of cirrhotic liver changes. Frequent association of cirrhosis with tumors can be explained by the fact that cirrhosis indicates previous liver damage and irregular regeneration. It is then necessary (1) to regard cirrhosis as a secondary process replacing the degenerated liver parenchyma; (2) to presume that a focal liver damage pre-existed in cases of tumors arising in non-cirrhotic livers. From the results of this experiment it is apparent that the initial parenchymal necrotic changes in the liver produced by the hepatotoxic action of 2-AAF invariably occurred prior to tumor formation. The percentage of liver tumors was considerably higher in males, which also were more susceptible than female rats to the hepatotoxic action of the compound. The incidence of liver tumors in male rats was higher when a larger dose was administered, although the total number of tumors in all organs was not appreciably changed. Therefore, it appears probable that the initial hepatotoxic action of higher dosages of 2-AAF has a localizing effect on the carcinogenic action of the compound.

Summary and conclusions. (1) Histological changes in the liver during the administration period of 2-acetylaminofluorene were studied by means of repeated partial hepatectomies. (2) It was observed that malignant liver tumors produced by 2-AAF are preceded by a certain pattern of changes in liver structure, beginning with subacute necrosis, nodular

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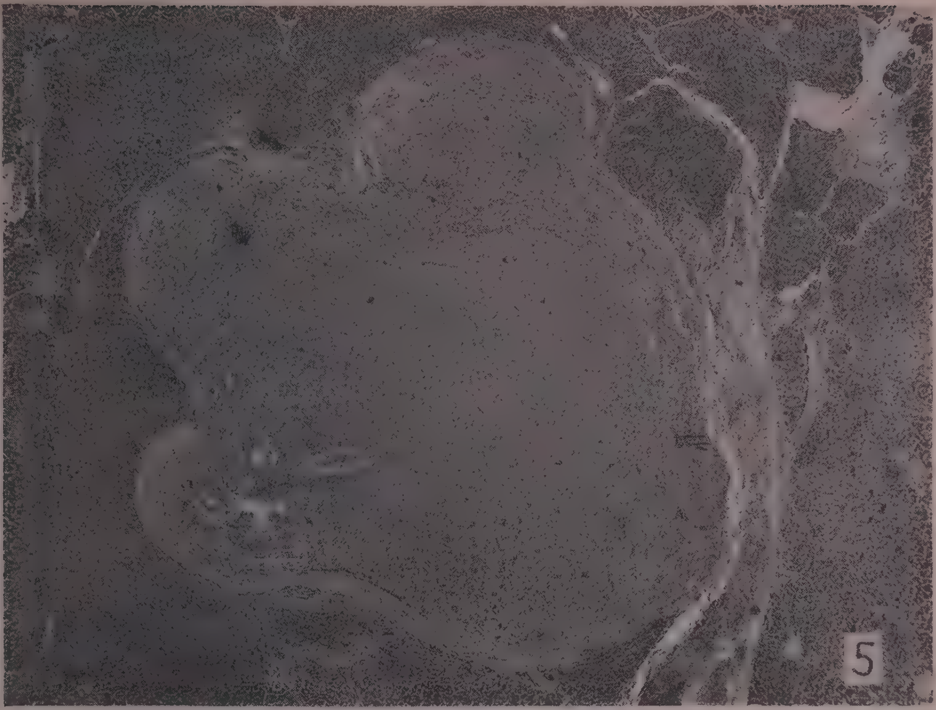

FIG. 5. Benign trabecular hepatoma. H & E. × 31.

hyperplasia, and cirrhosis. (3) Although these findings confirm the frequency of hyperplasia preceding malignant tumor formation, the actual conversion to malignancy does not seem to follow necessarily the condition of hyperplasia and/or benign neoplasia. (4) Repeated partial hepatectomies did not increase the incidence or accelerate the occurrence of hepatic tumors. (5) The percentage of liver tumors was considerably higher in males, which were found to be more susceptible than female rats to the hepatotoxic action of 2-AAF. (6) The incidence of liver tumors apparently depends on the production of hepatic damage, which has a localizing effect on the carcinogenic action of 2-AAF.

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Difference between Autoclaved and Sterile Filtered Medium in Culturing Fragments of a Marine Hydroid.* (18975)

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Invertebrates, especially those from the lower phyla which possess a high regenerative capacity, present interesting material for ex-

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[†] The author wishes to express her gratitude to Dr. Robert Chambers for the arrangements enabling the accomplishment of this work and for the stimulating discussion; to Dr. Philip R. White, in whose laboratory this work was done, for his valuable suggestions and for the opportunity of acquiring his excellent methods.

periments on redifferentiation of tissues and cells. Encouraged by the simplicity of the methods for tissue culture employed by Dr. Philip R. White and by the abundance of hydroids at The Mount Desert Island Biological Laboratory, the author decided to use a hydroid for experiments on the behavior of invertebrate cells *in vitro*.

Clava leptostyla, Agassiz(1), is a hardy hydroid common on the north Atlantic coast. It is found attached to Fucus plants and is able to survive many hours in the sea water clinging to the plants exposed at low tide. Clava is best cultured in an aquarium with running sea water or in dishes in which the sea water is changed every 48 hours. The stalk of Clava is smooth and in contrast to many other marine hydroids is not inhabited by commensals such as sessile protozoa and other microorganisms. For these reasons and because of its high regenerating power the author selected Clava as material for cultivation. Healthy looking organisms with long stalks were washed by repeated transfers (at least 10 times) in small Petri dishes filled with autoclaved sea water. After this preliminary washing the base of the stalk and the upper part of the body carrying the tentacles and the gonophores, were cut off with a sterile knife or scissors. The separated stalks were then picked up and again washed several times in autoclaved sea water. Finally the stalks were lifted out of the water and cut on a sterile dish into small pieces. By means of a sterile pipette the fragments were transferred to hanging drops of a liquid medium, 2 to 3 fragments for each drop, on a sterile coverslip and placed in a moist chamber. The moist chamber consisted of a glass ring fastened to a slide with vaseline. Only the coverslip, the medium and the pipette were sterile. Cultures were also made in roller tubes and in Carrel flasks under perforated cellophane. From preliminary experiments the use of penicillin (100 units per ml) was found to be superfluous since sterile conditions could be obtained without its use.

The fragments were selected in lengths of 0.5, 1.0 and 2.0 mm and were immersed in 3 types of media all at 17-22°C. The media were A, autoclaved sea water, B, sea water sterilized by filtration through a Selas porcelain filter and, C, a modification of White's synthetic medium, M & W(2,3).‡

The cells of the 0.5 mm fragments rounded up after a few hours and separated from one another. On the cut edges of the fragments most of the cells became highly vacuolated and increased in size. In Carrel flasks and roller tubes all cells underwent vacuolization. The individual cells survived 24 to 48 hours after which they disintegrated. Of the 2.0 mm fragments almost all the pieces closed over their cut ends. No further change occurred during the next 24 to 48 hours irrespective of the kinds of media and culture dishes used. The 1.0 mm fragments showed the most interesting results. In hanging drops containing autoclaved sea water a migration of endodermal cells started at both cut edges of the fragment after 16 hours. The migrating cells became oblong and narrow. The most characteristic feature was the formation of long projections. The slow progressive migration changed the endodermal layer after 4 days into dissociated cells with long projections. The ectodermal cells showed a tendency to migrate in sheets composed of cells with small projections along the free borders of the sheets. Such cultures were maintained in hanging drops for over two weeks. Subcultures of small groups of cells or single cells were made for observations on growth. Evidence of growth was very slight, possibly due to the poor nutritional quality of the medium, which consisted only of autoclaved sea water.

In hanging drops containing sea water sterilized by filtration or in modified White's

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1. Fraser, C. M., Hydroids of the Atlantic Coast of North America, 1944, Univ. of Toronto Press, Toronto, p16.

2. White, P. R., *Growth,* 1946, v10, 249.

3. White, P. R., *J. Cell. and Comp. Physiol.,* 1949, v34, 223.

‡ White's synthetic medium, M & W, used in these experiments was modified by increasing the concentration of the salts (NaCl, $MgSO_4$, $Ca(NO_3)$ HOH, KCl) threefold. The purpose of the modification was to adjust the osmotic value of White's synthetic medium to the osmotic value of sea water.

synthetic medium the 1.0 mm fragments behaved in an entirely different manner. The cells on the cut edges of the fragment rounded up and underwent vacuolization dissociation and disintegration. The rest of the fragment remained unchanged during the following few days. The results with those placed in Carrel flasks and roller tubes (in all kinds of used media) were similar to those in experiments with 0.5 mm fragments.

These experiments show that the behavior of the animal fragments depends not only on the size of the fragments but also on the medium and on the amount of culture fluid, which differed according to the kind of culture dishes used.

In comparing the different media it is of interest to note that migration of cells occurred only when the medium consisted of autoclaved sea water. This was surprising because it had been assumed that sterilization by filtration would be less damaging to the chemistry of the sea water than the prolonged heat of autoclaving. W. H. Johnson(4), working with sterile cultures of *Paramecium multimicronucleata* has also found media sterilized by autoclaving more satisfactory than those sterilized by filtration through a Seitz or a sintered glass filter.§ Possibly sterilization by filtration removes some essential components from the medium.

Summary. *Clava leptostyla,* Agassiz, a marine hydroid, is good material for obtaining separated and migrating cells in tissue culture. A thorough washing of fragments in autoclaved sea water was sufficient to provide bacteria-free conditions. The behavior of the cells depended on the size of the fragments, the kind of medium and the amount of culture fluid. Fragments about 2 mm in length tended to close over along the cut edges thus preventing migration of cells. In 0.5 mm fragments the cells rounded up, underwent vacuolization and distintegration after 24 to 48 hours. In fragments about 1 mm in length migration of endodermal cells was observed but only in hanging drops of autoclaved sea water. Cell migration was never observed in cultures containing sea water sterilized by filtration with a Selas porcelain filter.

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4. Johnson, W. H., *Proc. Am. Soc. of Protozool.,* 1950, v1, 9.

§ Personal communication.

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Free Amino Acids in Adult Mosquitoes.* (18976)

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Most of our information concerning amino acids in insects has been accumulated during recent years mainly through the application of the paper chromatographic method. Attention has been centered largely upon the amino acids occurring free in the hemolymph and other body fluids of insects in different stages of development(1,2). Such data have been obtained from a relatively small number of insect species representing only a few orders. In general these studies have been confined to large, immature forms which better lend themselves to analysis of body fluids.

The present work was undertaken as part of a study to elucidate factors which govern the susceptibility of mosquitoes to malarial parasites. Inasmuch as certain amino acids are known to be essential for *in vitro* growth and multiplication of malarial parasites, and since strains are known to differ in their amino acid requirements(3), it appeared that

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* This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

1. Agrell, I., *Acta Physiol. Scand.,* 1949, v18, 247.
2. Levenbook, L., *Biochem. J.,* 1950, v47, 336.

differences in the free amino acid content of various species of female mosquitoes might have some bearing upon their differential susceptibility or resistance to malarial parasites. It was therefore desirable to ascertain whether such differences actually existed.

Materials and methods. Laboratory-reared mosquitoes (*Culex quinquefasciatus, Culex pipiens, Aedes aegypti* and *Anopheles quadrimaculatus*) were used as well as *Culex salinarius, Aedes sollicitans* and *Culiseta inornata* which were collected in this vicinity as fourth stage larvae and pupae and reared to adulthood in water from their respective breeding habitats. Those species colonized in the laboratory were fed a standard diet consisting entirely of finely powdered dog chow. Unfed, adult female mosquitoes were collected 2 to 3 days after emergence and lightly anesthetized with chloroform. These were immediately weighed and homogenized in a tissue grinder with 1 ml of water. The material was centrifuged and the supernatant retained. Protein precipitation was carried out by adding one volume of 95% ethanol to about 5 ml of sample, and centrifuging. The supernatant was shaken with 3 volumes of chloroform (15 ml) with subsequent centrifugation at a low speed, and the aqueous fraction was removed for amino acid determinations. The paper chromatographic method was employed for qualitative and semi-quantitative determinations of free amino acids in the mosquito extracts. Whatman filter paper No. 4 was used with water-saturated phenol as the first solvent and 2,4-lutidine (3 parts to 1 part water) as the second. The quantity of extract usually delivered to the filter paper represented 12.5 mg (approximately 6 mosquitoes) for the one-dimensional chromatograms and 40 mg for the 2-dimensional runs. Amino acids and peptides were revealed by spraying the filter paper with a solution of 0.2% ninhydrin in water-saturated butanol and heating it for approximately 15 minutes at 80°C. Amino acids in pure form were added to various samples of unknowns for verification of preliminary identification based upon position and color of the spots appearing on the 2-dimensional chromatogram.

Results. The following amino acids were demonstrated in the extracts of all species of mosquitoes included in the present study: Alanine, glutamic acid, beta-alanine, isoleucine, leucine, taurine, proline, histidine, serine, valine, methionine, threonine, lysine, arginine, aspartic acid, glycine, tyrosine and tryptophan. Alanine and beta-alanine were present in relatively large amounts as indicated by the size and color of the spots. Glutamic acid and the leucines were less concentrated and the remaining amino acids appeared as much lighter spots indicating a comparatively low concentration. Although isoleucine and leucine were not usually completely separated on the typical chromatogram, this was accomplished by modification of the pH of various extracts. Tryptophan was represented by a very faint spot which could be seen immediately upon heating the ninhydrin-sprayed paper but disappeared soon thereafter. Although it was expected that cystine might be present, a small amount of hydrogen peroxide added to a portion of the various mosquito extracts revealed in only one instance a spot in the position of cysteic acid in the oxidized sample which was absent in the non-treated sample. A spot appearing immediately above beta-alanine, possibly a peptide, has not been identified due to considerable overlapping. This spot is blue when first seen, but it becomes purple in a matter of a few minutes.

Inasmuch as there were no apparent qualitative differences in free amino acids of various mosquito species, it was desirable to determine whether there were any consistent differences in the concentration of amino acids in the various genera and species as judged by parallel, one-dimensional chromatograms in phenol using standard amounts of extracts. A typical chromatogram of three genera of mosquitoes is seen in Fig. 1 (A-C). It will be noted that although there is only a slightly greater intensity of amino acid spots in *C. quinquefasciatus* than in *Aedes aegypti,* both of these culicine mosquitoes exhibit a considerably higher concentration of amino acids than *A. quadrimaculatus.* In contrast to these

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3. Geiman, Q. M., Proc. 4th Int. Cong. Trop. Med. Mal., 1948, v1, 618.

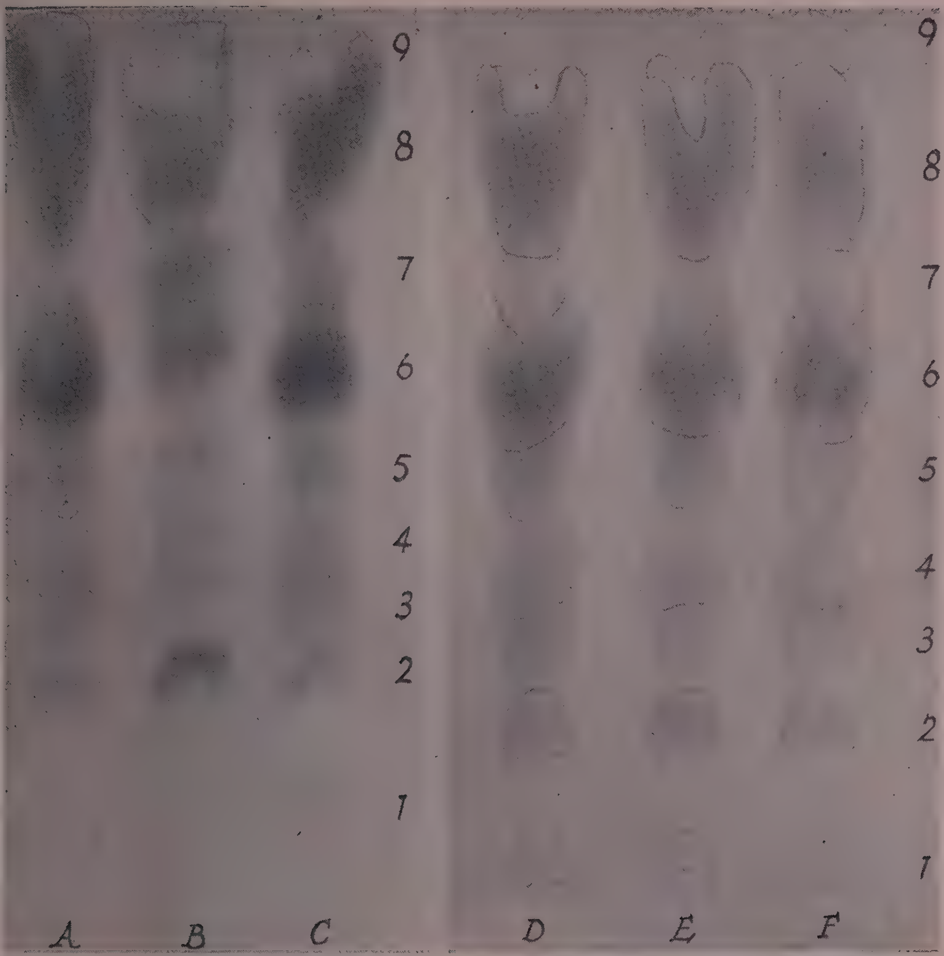

FIG. 1. Chromatograms of ethanol extracts. A, *Aedes aegypti*, B, *A. quadrimaculatus* and C, *C. quinquefasciatus*. (1, aspartic acid; 2, glutamic acid; 3, taurine; 4, serine and glycine; 5, threonine and lysine; 6, tyrosine, alanine and beta-alanine; 7, valine and arginine; 8, tryptophane, leucines, methionine and histidine; 9, proline.) D, *C. pipiens*, E, *C. quinquefasciatus* and F, *C. salinarius*. (1-9 as above).

intergeneric variations semi-quantitative differences between species belonging to a single genus were much less marked. The consistency of the amino acid pattern in 3 species of *Culex* mosquitoes is indicated in Fig. 1 (D-F). Except for the absence of a spot in the position of aspartic acid for *C. salinarius*, intraspecific differences are practically negligible. It is interesting, however, to note the striking similarity between the patterns of *C. pipiens* and *C. quinquefasciatus* since these mosquitoes though differing in their geographic distribution and in certain morphological characters are thought to be subspecies(4).

Extracts of *A. aegypti* and *A. sollicitans* were compared in a similar fashion and again, only slight differences in the intensity of certain spots were noted.

Discussion. It is apparent from the foregoing results that adult, female mosquitoes, like other insects studied, have a relatively high free amino acid content in their body

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4. Farid, A. F., *Amer. J. Hyg.*, 1949, v49, 83.

fluids. Other members of the order Diptera have been investigated; however, immature stages were used in order that the hemolymph *per se* could be extracted in sufficiently large quantities for analysis. Agrell(1), working with *Calliphora erythrocephala* pupae, demonstrated the presence of all the amino acids found in the present study with the exception of histidine and tryptophan. He also reported beta-alanine, taurine and methionine for the first time, all of which are present in adult mosquitoes. It is interesting to note that alanine, which is present in high concentrations in adult mosquitoes, likewise was predominate in both *Calliphora erythrocephala* larvae(5) and pupae(1). Although Golberg and DeMeillon(6) found that cystine was a necessary constituent of the diet of *Aedes aegypti* larvae, the present study suggests that it does not occur in free form in the body fluids of the adult. Tryptophan has not previously been reported for any of the Diptera studied. Phenylalanine also appears to be an uncommon constituent of insect hemolymph and other body fluids, having been reported only from larvae of the order Diptera by Finlayson and Hamer(5) in *Calliphora erythrocephala* and by Levenbook (2) in *Gastrophilus intestinalis*. According to Golberg and DeMeillon(6) it is an essential dietary constituent for *Aedes aegypti* larvae. It is the only amino acid of those found by them to be essential for these mosquito larvae that was not detected in the extracts of adult mosquitoes.

Summary. The free amino acids of adult, female mosquitoes (*Culex pipiens, Culex quinquefasciatus, Culex salinarius, Aedes aegypti, Aedes sollicitans, Culiseta inornata* and *Anopheles quadrimaculatus*) were extracted with ethanol and identified by paper chromatography. Extracts of all species of mosquitoes contained 18 amino acids: Alanine, glutamic acid, beta-alanine, isoleucine, leucine, taurine, proline, histidine, serine, valine, methionine, threonine, lysine, arginine, aspartic acid, glycine, tyrosine and tryptophan.

Extracts of *C. quinquefasciatus* and *A. aegypti* exhibited relatively larger amounts of most amino acids than those of *A. quadrimaculatus* as indicated by the size and intensity of spots on the chromatograms. Such differences between 3 species of *Culex* mosquitoes and 2 species of *Aedes* were practically negligible.

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Tumor Transplantation to the Subdural Space of Heterologous Hosts.*
(18977)

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It is not known why the anterior chamber of the eye of heterologous hosts is a suitable site for tumor transplantation. The experiment to be reported was devised to determine whether or not the subdural space of guinea pigs is as favorable a transplantation site for Mouse Sarcoma 37 as the anterior chamber of the eye. Following transplantation to the anterior chamber, grafts of this tumor have been found to become subtotally necrotic within 24 hours. Cells from the periphery of the graft invade the iris and the pectinate ligament. Here they multiply and become vascularized by the host. Growing tumor has

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* Aided by a Grant from the National Cancer Institute, U. S. Public Health Service.

been found in all guinea pigs between the 6th and the 11th day after transplantation to the anterior chamber(1). Regression of tumor tissue invariably takes place, when, in the great majority of animals, only a small portion of the anterior chamber contains tumor tissue. Regression begins at the end of the 2nd week, and is completed within the 3rd week after transplantation. Growth of this tumor has not been observed following subcutaneous transplantation to guinea pigs. The subdural space was selected because it resembles the anterior chamber in the following respects: (1) it contains clear fluid (similar to the cerebrospinal fluid), which drains directly into the blood stream (*cf.* canal of Schlemm); (2) the normal fluid in both spaces is apparently low in protein; (3) both spaces are limited on one surface by a relatively avascular fibrous membrane, and on the other, by a highly vascular reticular tissue; (4) both the iris and the arachnoid develop from mesenchyme in the immediate vicinity of the brain.

Material and methods. Mouse Sarcoma 37, obtained from the National Cancer Institute, has been carried in this laboratory for 58 transplant generations by subcutaneous inoculation of mice of the ABC strain. Adult guinea pigs of either sex were obtained locally, and fed a commercial diet regularly supplemented with ascorbic acid. The transplantations to the subdural space were done as follows: The fronto-parieto-occipital area was depilated (barium sulfide). A curved incision was made extending from behind the right ear to the midline and down to the right eye. A rectangular bone flap (about 1.0 x 0.8 cm) was cut from the right parietal region, (using a portable, electric disc saw), leaving the dura intact. The bone flap was lifted medially, leaving it hinged laterally. A 2 mm slit was made in the inferomedial corner of the dura, and a tumor-laden trocar (No. 17 spinal needle) inserted into the subdural space. The tumor was deposited under the semi-transparent dura in the diagonally opposite corner. The hinged bone flap was closed, and the skin approximated with continuous No. 60 cotton suture. The animals were anesthetized with ether. Cotton applicators moistened with penicillin solution (approximately 25,000 units/cc) were used for sponging. The procedure presented little difficulty when care was applied to avoid hemorrhage on approaching the dura. It lasted less than five minutes and was withstood well by all animals. There were no post-operative deaths. The animals were sacrificed with ether and by exsanguination. The area beneath the bone flap, consisting of dura and subjacent tissue (tumor, arachnoid and brain) was removed *en bloc* and fixed in 10% formalin.

Experiment. Mouse Sarcoma 37 was transplanted to the subdural space of 14 guinea pigs. The animals were sacrificed after 10 hours, and on the 1st, 2nd, 3rd, 5th, 7th, 9th, 11th, 14th, and 18th day after transplantation. One animal died on the 21st day.

The subdural transplantation sites were readily identified during the first 5 days as a hemorrhagic area in the region of the original graft. Microscopically the bulk of the tumor had become necrotic by the first day and free tumor cells were found to penetrate the arachnoid and spread through the subarachnoid space. Growing tumor was found in all animals sacrificed thereafter.

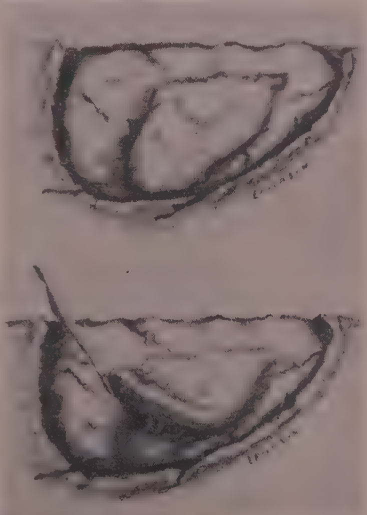

FIG. 1. Mouse Sarcoma 37, 11 days after transplantation to subdural space of guinea pig.

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[1] Eichwald, E. J., and Chang, H. Y., *Am. J. Path.*, in press.

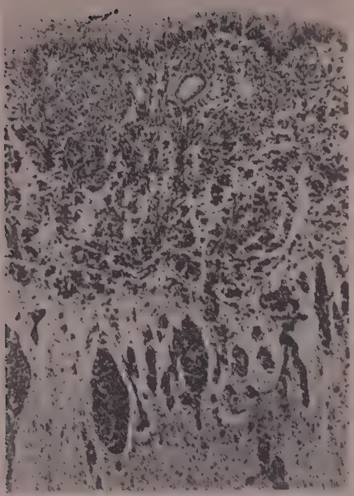

FIG. 2. Mouse Sarcoma 37, 14 days after transplantation to subdural space of guinea pig.

By the end of the 1st week a solid layer of tumor tissue was present in the subarachnoid space, and rows of tumor cells infiltrated the brain along the perivascular spaces. Areas of necrosis and fibrosis were present in the upper layers of the tumor. The animal sacrificed on the 11th day showed an ovoid polypoid meningeal mass measuring 1.1 x 0.8 x 0.4 cm, lying upon and depressing the brain surface (Fig. 1). On the 14th and the 18th day there was deep invasion of the brain with gross extension to the proximity of the ventricle and thick cuffs of tumor cells around the cerebral vessels (Fig. 2). The tumor of the animal sacrificed on the 18th day measured 1.5 x 1.0 cm over its surface and extended 0.4 cm into the brain tissue. This is 3 to 4 times larger than the largest size obtained in this laboratory following transplantation of this tumor to the anterior chamber and several hundred times larger than the average size. The last surviving animal with subdurally transplanted tumor developed a gait disturbance during the 3rd week and died 21 days after transplantation. The autopsy showed hemorrhage and necrosis of the brain and abundant well preserved tumor tissue. No lesions of note were seen in the other viscera.

Summary. Mouse Sarcoma 37 was transplanted to the subdural space of 14 guinea pigs. Evidence of growth was present in all animals sacrificed between the 2nd and 18th day after transplantation. One animal died at the end of the third week, apparently as the result of invasion of the brain by tumor tissue. The pattern of growth in the meninges and brain was similar to that previously observed in the anterior chamber of the eye of guinea pigs, but, after the 9th day the mass of tumor tissue in the brain was greater than that in the eye for comparable periods. There was no evidence of regression of tumor in the brain in contrast with tumor transplanted to the anterior chamber, which usually begins to regress by the end of the 2nd week.

Since this paper was submitted for publication, H. S. N. Greene published an excellent paper on "The Transplantation of Tumors to the Brain of Heterologous Species," *Cancer Research*, 1951, v11, 529.

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Depressor Effect of Potassium Restriction on Blood Pressure of the Rat.
(18978)

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Although there is ample evidence(1,2) that an adequate intake of potassium is necessary for the structural integrity of the cardiovascular system, almost no hemodynamic studies

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1. Schrader, G. H., Prickett, C. O., and Salmon, W. D., *J. Nutrition,* 1937, v14, 85.

2. Follis, R. H., Jr., Orent-Keiles, E., and McCollum, E. V., *Am. J. Path.,* 1942, v18, 29.

of the potassium deficient animal have been made. Recently, however, in a preliminary communication(3) we reported the occurrence of hypotension in a group of rats placed upon a diet containing only 0.01% potassium. In the present report these preliminary studies have been extended, and the influence of sodium intake upon the depressor effect of potassium restriction also investigated. The results of these studies not only confirm the preliminary finding concerning the hypotensive effect of potassium restriction, but also suggest that the lowering of the blood pressure can be prevented by the simultaneous restriction of sodium in the diet.

Methods. Male rats (Long-Evans strain), 45 days old, were used in all experiments. Variations in either the potassium or sodium intake were made by adding known amounts of either cation to an otherwise basic diet, as described by Follis *et al.*(2), consisting of known and fixed amounts of carbohydrate, fat, protein, salts, and vitamins. These values were checked by analysis using the flame photometer method. Blood pressure readings were taken by the microphonic method(4) before the experiment and then once weekly for 7 weeks. All rats were killed 8 weeks after the beginning of the experiment. The heart, kidneys and adrenal glands of the various groups of rats were removed and weighed (dry and wet). Histological studies also were made of the same organs.

Results. A. *Effect of dietary potassium restriction upon blood pressure of rats.* Twenty-nine rats were placed upon the basic diet containing 0.01% potassium and 0.4% sodium. Twenty-four of these rats were living at the end of the 7-week period. The average weight of the group at the beginning and end of the experimental period was 124 g (range: 96 to 177) and 162 g (range: 108 to 222), respectively. The average initial blood pressure of the group was 104 mm Hg (S.E. mean: ±2.3). The average pressure slowly decreased until at the end of the 7th week the average pressure was 84 mm Hg (S.E. mean: ±2.1). The rats appeared to be in fairly good health, but when they were held in the hand it was apparent that they were asthenic and had poor muscle tonus. Although they moved about freely, they lacked vigor.

A control group of 29 rats was given the same diet containing, in addition, 0.6% potassium. These rats gained much more weight; their average initial weight of 113 g (range: 101 to 128 g) increased to 234 g (range: 201 to 273). The course of their blood pressure measurements also was different. Thus, the average initial pressure of 105 mm Hg (S.E. mean: ±1.9) increased significantly over 7 weeks to a final reading of 113 mm Hg (S.E mean: ±2.1), as might be expected in growing rats.

B. *Effect of sodium intake upon hypotension induced by dietary potassium restriction.* In view of the above depressor effect induced by the dietary restriction of potassium, it was considered important to determine the possible influence of concomitant alterations in the sodium intake upon this response. Therefore, one group of 21 rats was placed upon low potassium diet (0.01%), which also contained a low sodium content (0.04%). The weight and blood pressure of both groups were followed weekly for 7 weeks. An actual fall in weight occurred in the group of rats maintained upon the low sodium-low potassium diet. Thus, the average initial weight was 117 g (range: 89 to 164) and the average final weight was 111 g (range: 73 to 178). No fall occurred in the blood pressures of these

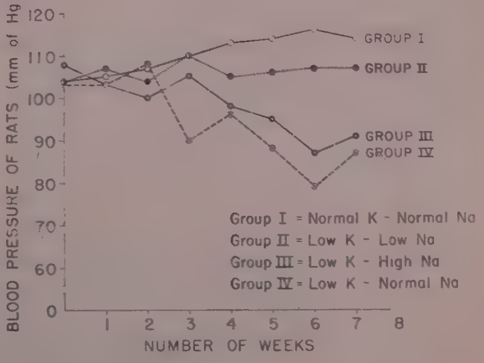

FIG. 1.

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3. Freed, S. C., and Friedman, M., *Science*, 1950, v112, 788.

4. Friedman, M., and Freed, S. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 670.

TABLE I. Average Wet and Dry Weight of Kidneys, Heart and Adrenals in Potassium Deficient Rats.*

Diet		No.	Kidneys			Heart			Adrenals		
K	Na	rats	Wet, g	Dry, g	W/D ratio	Wet, g	Dry, g	W/D ratio	Wet, g	Dry, g	W/D ratio
Low–	Normal	10	1.36	.32	4.3	.38	.08	4.6	.017	.010	1.7
’’	Low	17	1.12	.28	4	.41	.10	4.1	.027	.014	1.8
’’	High	19	1.32	.33	4	.40	.095	4.2	.020	.008	2.5
Controls		6	.82	.27	3	.39	.12	3.3	.018	.010	1.8

* Calculated per 100 g body wt.

rats over the period of the experiment. Thus, the initial average pressure was 104 mm Hg (S.E. mean: ±1.6) and at the end of 7 weeks the average pressure was 107 mm Hg (S.E. mean: ±4.5) The animals, however, presented the same general appearance of asthenia observed in the previously described group of rats which were restricted *only* in their potassium intake. Twenty-two rats placed upon a low potassium diet containing a high sodium content (0.70%) exhibited relatively little gain in weight (increase of 130 to 142 g over the 7-week period). The fall in blood pressure was somewhat less than that found in rats receiving a normal amount of sodium in their low potassium diet. Their average initial pressure was 108 mm Hg (S.E. mean: ±2.2) and at the end of 7 weeks it was 91 mm Hg (S.E. mean: ±3.1).

C. *Pathological changes following dietary potassium restriction.* The heart, kidneys and adrenal glands of the various groups of rats were examined histologically at the end of 7 weeks. Rats placed upon diets low in potassium but normal or high in sodium invariably exhibited foci of necrosis and cellular infiltration in the myocardium. These lesions were found chiefly in the subendocardial area of the left ventricle. Although all of the potassium deficient rats exhibited these lesions, no correlation could be found between the severity of histological change and degree of depressor effect. Rats placed upon a diet not only low in potassium, but also low in sodium did not exhibit degenerative changes in the myocardium.

From Table I, it can be noted that kidneys of the animals on low potassium diets with varying sodium content were heavier than normal. This increased weight, however, was due to a greater water content, as indicated by the essential equivalence of the dry weights to that of the control group. The reverse relationship occurred with the heart weights, the wet weights being comparable to the controls, but the dry weights being somewhat decreased. This latter suggests the possibility that rats on the low potassium diets had a decreased myocardial mass. The adrenal glands of the rats on the low potassium-high sodium diet were apparently edematous while those on the low potassium-low sodium diet were possibly hypertrophic. These results are essentially in confirmation of the findings of Kornberg and Endicott(5) who reported widespread edema in rats on a low potassium diet.

Discussion. The foregoing observations strongly suggest that drastic restriction in the rat's intake of potassium leads to a gradual fall in the animal's blood pressure. The exact mechanism responsible for the observed hypotension is still not known. However, the general inanition resulting from potassium deprivation cannot be held responsible, because control animals either partially starved(3) or those markedly emaciated from feeding on the diet low in sodium as well as potassium did not exhibit any significant change from normal in their blood pressure. Moreover, no correlation could be found between the severity of the myocardial necrosis occurring in the potassium deficient rat and degree of hypotension. Studies concerning the mechanism of the lowering of blood pressure are being pursued. The prevention of the depressor effect of the potassium deficient diet by the withdrawal of dietary sodium was an interesting, but not necessarily a surprising, phenomenon. It is well known that sodium displaces potassium in the tissues(6). It can

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5. Kornberg, A., and Endicott, K. M., *Am. J. Physiol.*, 1946, v145, 291.

be assumed that in the presence of a low sodium content in the diet, the potassium in the tissues is retained sufficiently to prevent the development of hypotension. Biochemical studies will be required to prove this point.

Summary. (1) Dietary restriction of potassium was found to lead to the occurrence of hypotension in the rat. (2) This depressor effect of potassium deprivation was prevented by simultaneous dietary withdrawal of sodium.

The authors wish to express their gratitude for the valuable assistance of Dr. Sanford O. Byers.

Received July 13, 1951. P.S.E.B.M., 1951, v78.

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Effect of Variation of Potassium Intake on Pressor Activity of Desoxycorticosterone. (18979)

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Although the chronic administration of DCA may induce hypertension in various animals, it occurs regularly in the rat only if rather large doses are administered to younger animals(1). Furthermore, hypertension occurs uniformly only when additional sodium is given and when renal damage or uninephrectomy has been accomplished(1-3). Even under many of these conditions, certain investigators have not been able to produce a significant hypertension(1,3,4). Sayers(1) concluded that "the impression is gained that DCA must act in combination with as yet unknown factors in the production of pathological lesions in experimental animals." One of the possible factors responsible for the irregularity in the frequency, intensity, and persistence of the hypertension induced by DCA is the potassium loss induced by this steroid(5). For loss of this cation has been found in our laboratory to produce a marked hypotension in both the normotensive(6,7) and the hypertensive(8) rat. In other words, DCA may initiate one or more mechanisms to elevate the blood pressure, yet also initiate one (*e.g.* the loss of potassium) to decrease the blood pressure.

In view of the latter possibility, it was thought worthwhile to study the effect of DCA in the rat deprived of potassium and in the rat fed an excess of this same cation. The results of the study indicated that the effects of DCA on the blood pressure were far more profoundly influenced by experimental variation of the potassium intake than that of sodium.

Effect of DCA on rats deprived of potassium. Methods. A group of 24 six-weeks-old male rats (Long-Evans) was deprived of potassium by placing them on a potassium-deficient diet(8). At the end of 8 weeks, 8 of these potassium-deficient rats were given DCA in benzyl alcohol† (4 mg) 3 times a week by

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* Research Fellow of the Amer. Heart Assn.

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3. Knowlton, A. I., Stoeric, H., Seegal, B. C., Loeb, E. N., *Endocrinol.*, 1946, v38, 315.

4. Friedman, S. M., Polley, J. R., and Friedman, C. L., *J. Exp. Med.*, 1948, v87, 329.

5. Gaunt, R., Birnie, J. H., and Eversole, W. J., *Physiol. Rev.*, 1949, v29, 281.

6. Freed, S. C., and Friedman, M., *Science*, 1950, v112, 788.

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8. Friedman, M., Rosenman, R. H., and Freed, S. C., *Am. J. Physiol.*, in press.

† We are grateful to Dr. Abbott E. Allen of the Schering Corp. for generous supplies of DCA used in these studies.

subcutaneous injection. Eight more were treated in the same fashion, except that they also drank sodium chloride solution (1%). The remaining 8 rats received no DCA but were maintained on the potassium-deficient diet as controls. Systolic blood pressures were taken weekly by means of the microphonic manometer(9).

Results. The rats deprived of potassium had an average blood pressure of 84 mm Hg (range: 60 to 98 mm) at the end of the 8-week period of feeding a potassium-deficient diet. The administration of DCA induced an even more marked depressor response, the average blood pressure falling to 65 mm Hg (range: 50 to 80 mm) within one week. Marked loss of weight occurred in these animals, all of which died in less than 3 weeks. Moreover, those rats drinking saline solution showed a more marked decline in blood pressure, to an average of 60 mm Hg, and even greater toxicity, death occurring in all by the end of the first week. In the control potassium-deficient rats, the average blood pressure remained at the same hypotensive levels; moreover, no loss of weight or mortality occurred in this group.

Effect of excess potassium compared to excess sodium in intact rats given DCA. Methods. Sixteen 6-weeks-old male rats (Long-Evans) were divided into 2 groups. The first group of 8 was fed a normal diet but received potassium chloride (1%) to drink. The remaining 8 rats were fed the same diet but received sodium chloride (1%) to drink. DCA in benzyl alcohol (4 mg) was administered 3 times a week by subcutaneous injection to all of the rats. Blood pressures were obtained at weekly intervals.

Results. As Fig. 1 shows, both groups of rats exhibited an increase in blood pressure following DCA injections. However, those DCA-treated rats drinking KCl solution showed an increasingly higher degree of hypertension in sharp contrast to those drinking NaCl solution which demonstrated a decline in blood pressure starting at the sixth week and eventually becoming normotensive.

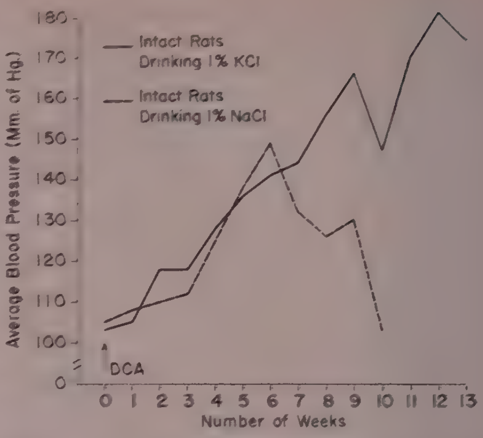

FIG. 1. The effect of DCA on intact rats given supplemental potassium or sodium.

The gain in average weight was greater in those rats given the excess potassium (178 to 315 g) than in those drinking sodium chloride solution (184 to 273 g). Deaths, as well as gross and microscopic evidence of toxicity were noted only in the latter group.

Discussion. The results of the preceding studies indicate that the pressor effect of DCA can be either abolished or accentuated in the normal rat with intact kidneys by the removal or addition, respectively, of potassium to the dietary intake of the experimental animal. Indeed, DCA was found to exert a further depressor effect in rats previously made potassium-deficient. Since it was shown previously that potassium deprivation has a depressor effect in both normotensive and hypertensive rats(6,8), this depressor effect of DCA may well be related to the loss of potassium which occurs during administration of this steroid(5). The loss of potassium occasioned by DCA is intensified by the concomitant administration of excess sodium(10) and would seem responsible for the more marked hypotension which occurred in the rats previously made potassium-deficient, as well as for the decline which followed the initial elevation of the blood pressure in the intact rats drinking saline (Fig. 1).

It is significant that when excess potassium

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9. Friedman, M., and Freed, S. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 670.

10. Peschel, E., Black-Schaffer, B., Schleyer, C., *Endocrinol.*, 1951, v48, 399.

was given, sustained hypertension developed in older rats with intact kidneys, and with relatively small dosage of DCA. Moreover, none of the latter rats died during the experimental interval, nor was gross evidence of toxicity observed in these animals, confirming the protective action of potassium in the DCA-treated animal(10-12).

Although most all of the pressor and toxic actions of DCA heretofore have been studied in reference to the cation, sodium, the importance of potassium in this same relation cannot be overlooked. Indeed, both these, as well as our earlier findings(8), strongly suggest that the presence of adequate amounts of the latter cation is essential for the development of hypertension in the rat, whether the latter is induced by the administration of DCA or by interference with renal hemodynamics.

Summary. Significant hypertension was produced by DCA in intact, older rats given excess potassium, in contrast with the failure of hypertension to persist in similarly treated rats but receiving excess sodium. Potassium deficiency abolished the pressor action of DCA in rats. The results of these studies also indicated that the effects of DCA on the blood pressure were far more profoundly influenced by experimental variation of the potassium intake than that of sodium.

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Work Performance of Adrenalectomized Rats Given Cortisone and 17-hydroxycorticosterone by Continuous Intravenous Injection. (18980)

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The biologic activity per unit weight of 17-hydroxycorticosterone has been found to be greater than that of 17-hydroxy-11-dehydrocorticosterone (cortisone) when assayed by the Ingle work-test(1,2) and by the liver glycogen deposition test(2). All biologic assays of the adrenal cortical steroids have been based upon the response to intermittent injection. Since there are some differences in the physical properties of the steroids the possibility must be considered that the apparent differences in potency are based upon differences in the rate of absorption from the site of injection or in the speed of inactivation of the compound after it enters the body fluids. Accordingly we have determined the biologic response per unit weight of 17-hydroxycorticosterone and of cortisone by administering each steroid by continuous intravenous injection to rats under the conditions of the Ingle work-test. In these experiments 17-hydroxycorticosterone had approximately twice the potency of cortisone.

Methods. Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they reached a weight of 200 ± 2 g. The work test was carried out according to Ingle(3). The animals were anesthetized with phenobarbital sodium and cyclopal sodium and were subjected to the stimulation of muscle immediately following removal of both adrenal glands and both kidneys. The quadriceps muscle of the left hind leg was weighted with 100 g. A Nerve Stimulator, Model B, Upjohn, was used to deliver 5 pulses per second. The duration of each pulse was 20 milliseconds and the intensity was 20 milliamperes. The distance the weight was lifted was recorded on automatic work adders. Each recorder revolution represented approxi-

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1. Ingle, D. J., and Kuizenga, M. H., *Endocrinology*, 1945, v36, 218.

2. Pabst, M. L., Sheppard, R., and Kuizenga, M. H., *Endocrinology*, 1947, v41, 55.

3. Ingle, D. J., *Endocrinology*, 1944, v34, 191.

TABLE I. Work* Performance of Adrenalectomized-Nephrectomized Rats Given Adrenal Cortical Steroids by Continuous Intrav. Inj. Total recorder revolutions. Averages and stand. dev. of averages.

Dose steroid, mg/24 hr/rat	Cortisone		17-hydroxycorticosterone	
	No. of rats	Total work	No. of rats	Total work
.1	15	11051 ± 700	15	13732 ± 863
.25	15	12396 ± 1447	15	22975 ± 1625
.5	15	21192 ± 1855	15	26408 ± 1716
1	16	28515 ± 2665	16	35074 ± 2027

* Each recorder revolution represents approximately 400 g-centimeters of work.

mately 400 gram-centimeters of work. Stimulation was continued until muscular responsiveness was lost. The animals were enclosed in a temperature cabinet with the temperature constant at 28 ± 0.5°C. The two steroids were each dissolved in water containing 5% ethanol, 0.9% sodium chloride and the potassium salt of crystalline penicillin G (Upjohn). The fluid load was 20 cc per 24 hrs per rat and the penicillin dose was 5000 units per 24 hrs per rat. The injections were made into the jugular vein by means of a continuous injection machine which was incorporated into the fatigue apparatus. By means of this apparatus 12 rats can be studied simultaneously.

Experiments and results. The two compounds were compared at dosage levels of .1, .25, .5 and 1.0 mg per rat per 24 hrs. The compounds were equally represented in each individual experiment. Each position on the fatigue apparatus and each automatic recorder was represented equally among the rats receiving each compound.

The group averages and the standard deviations of the averages are summarized in Table I. At each dosage level the average amount of work done by the rats treated with 17-hydroxycorticosterone was significantly greater than the average amount done by the rats treated with cortisone. In these experiments the potency per unit weight of 17-hydroxycorticosterone appears to be about twice that of cortisone, *i.e.*, in order to duplicate the work performed at a given dose of 17-hydroxycorticosterone it was necessary to give twice as much cortisone. This estimate may differ from the true ratio of activity due to errors in sampling.

Discussion. These data confirm the earlier observations(1,2) that 17-hydroxycorticosterone is more potent than cortisone per unit weight in supporting the ability of the adrenalectomized rat to work. The difference in quantitative activity is not due to differences in absorption into the body fluids for it remains evident when these steroids are delivered directly into the blood. The possibility remains untested that the quantitative differences in activity are based upon differences in the rate and extent of entry of the steroid from the blood into tissue cells.

The potency of any biologically active compound must always be defined in terms of the conditions under which it is tested. A biologic activity is not a quality which is determined solely by the compound itself but rather is a sign of an interaction with a responsive tissue. Consequently, ratios between the quantitative activities of two or more compounds may be significantly altered by changes in experimental conditions or may even be reversed. The greatest practical interest in 17-hydroxycorticosterone at the present time centers upon the possibility that it may be more effective than cortisone in treating the so-called connective tissue diseases. Although Thorn(4) and his associates have found that 17-hydroxycorticosterone is more effective than cortisone in eliciting eosinopenia in man and Conn(5) has found that 17-hydroxycorticosterone is the more potent of the two compounds in eliciting metabolic changes in man, the preliminary comparisons of the efficacy of the two compounds in the treatment of rheumatoid arthritis have thus far failed to support the hope that 17-hydroxy-

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4. Thorn, G. W., and Forsham, P. H., personal communication.

5. Conn, J. W., personal communication.

corticosterone would be superior to cortisone (6).

Summary. Under the conditions of these experiments 17-hydroxycorticosterone was found to have about twice the biologic potency of cortisone in the work performance test on adrenalectomized-nephrectomized male rats.

We wish to express our appreciation to Dr. Augustus Gibson, The Medical Division of Merck and Co., who supplied the cortisone and to Dr. W. J. Haines, Endocrinology Department, The Upjohn Research Laboratories, who supplied the 17-hydroxycorticosterone for these studies.

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6. Sprague, R. G., personal communication.

Nutritive Value of Legume Seeds. XI. Counteracting the Growth Inhibitor of Raw Soybeans.[*] (18981)

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The existence of a distinct growth-inhibiting factor for the chick in raw soybeans was demonstrated by Ham, Sandstedt, and Mussehl (1). That the soybean growth inhibitor similarly affects the rat has been established subsequently in several reports. One of the objectives of the present writers' investigations of the soybean growth inhibitor (SGI[†]) has been to answer this question: Is there a factor which will counteract the growth inhibitor of raw soybeans? Results of a search for an anti-SGI[†] are here described.

Experimental. The substances to be tested for anti-SGI activity replaced an equal weight of starch in the following ration: 25 g autoclaved or raw soybean oil meal, 0.6 g DL-methionine, 2 g salt mixture(2), 0.5 g choline chloride, 0.5 mg thiamine HCl, 0.5 mg Ca pantothenate, 2.5 mg niacin, 0.125 mg pyridoxine HCl, 0.5 mg riboflavin, 5 g cod liver oil, 15 g lard, and corn starch sufficient to make 100 g. The raw soybean oil meal was prepared with a minimum of heat treatment by the solvent process according to the manufacturer's statement. The autoclaved soybean oil meal was prepared from this by autoclaving at 15 lb pressure for 30 minutes. The rations were fed to weanling rats of the Sprague-Dawley strain. Animals of each litter were paired as to sex and initial weight between the 2 experimental rations, autoclaved soybean oil meal plus the material being tested for anti-SGI activity versus raw meal plus the same material. The rats were housed in individual screen-bottom cages with food and water available *ad libitum*. The number of rats fed each ration ranged from 8 to 20, the feeding period from 20 to 42 days. Some of the materials tested for anti-SGI activity are listed in Table I with the average gain per day at the end of 20 days feeding. Longer feeding periods did not affect the results other than to increase statistical significance. Of some 25 materials tested only trypsin powder has been found to be effective in counteracting the growth inhibitor in raw soybeans. Because of the interest in the trypsin inhibitor of raw soybeans, the apparent trypsin inhibitor activity of a mixture of 25 g raw soybean oil meal and 5 g trypsin

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[*] Published with the approval of the Director as Paper No. 539, Journal Series, Nebraska Agricultural Experiment Station. This work was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service. A preliminary report of some of these data was submitted in an abstract to the American Institute of Nutrition, Cleveland, May, 1951.

1. Ham, W. E., Sandstedt, R. M., and Mussehl, F. E., *J. Biol. Chem.*, 1945, v161, 635.

[†] It is proposed that the abbreviation "SGI" be used for the soybean growth inhibitor until identification has been accomplished. A factor which counteracts the growth inhibitor will be termed "anti-SGI."

2. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1937, v14, 273.

TABLE I. Anti-SGI Activity of Several Materials.

Material added	Rat growth g/day ± SE g gain/g food consumed Soybean oil meal Autoclaved	 Raw	Raw autoclaved, %
10% cow manure	3.32 ± .20 (.39)	1.79 ± .14 (.28)	54
5% fish solubles	3.47 ± .10 (.46)	2.55 ± .15 (.33)	73.5
Whey, 75 ml/100 g ration	3.94 ± .14 (.48)	2.90 ± .21 (.40)	73.6
0.1% Xanthine, 2 mg α-tocopherol in oil/day	2.83 ± .14 (.43)	2.10 ± .13 (.31)	74.3
5% yeast	3.63 ± .17 (.49)	2.70 ± .20 (.36)	74.4
None	3.27 ± .14 (.45)	2.51 ± .19 (.32)	76.7
0.5% L-arginine, HCl	3.91 ± .20 (.49)	3.08 ± .15 (.43)	78.8
5% whole liver powder	3.54 ± .19 (.50)	2.83 ± .10 (.42)	80
0.2% MK-42*	3.09 ± .19 (.47)	2.53 ± .13 (.42)	81.8
5% casein, tech.	3.69 ± .15 (.48)	3.08 ± .07 (.41)	83.5
5% trypsin powder†	3.44 ± .24 (.50)	3.57 ± .12 (.47)	103.9

* MK-42 stated to contain 12.5 mg vit B_{12} activity and 30 g streptomycin base per lb., courtesy of Dr. M. A. Schooley, Merck & Co., Rahway, N. J.

† Trypsin powder (1-110), Pfanstiehl Chemical Co., Waukegan, Ill.

powder, the proportions as fed, was determined by the method of Borchers(3). The trypsin inhibitor activity of the extract from a mixture of raw meal and trypsin was 32.4 × 10^{-3} units per ml, the activity of the extract from raw meal alone was 40.8 × 10^{-3} units per ml.

The following experiments were carried out in an effort to define some of the characteristics of the anti-SGI factor in the trypsin powder. The anti-SGI activity remained after the dry trypsin powder was autoclaved with live steam at 15 lb for 30 minutes. Autoclaving in this manner destroyed the trypsin activity as determined by the method of Anson(4). The anti-SGI activity was stable during autolysis when suspended in 10 ml water per g and incubated at 37°C and pH 8.5 for 48 hours, the activity being present in the soluble portion. Suspending the powder in 10 ml water per g, adding HCl to pH 3, adding ethanol to 60% concentration, and centrifuging yielded the active product in the centrifugate. The following tests were made on the 60% alcohol centrifugate after concentration *in vacuo* to remove the alcohol and diluting so that one ml was equivalent to 0.1 g original trypsin powder. Continuous extraction with n-butanol failed to remove any of the activity. Addition of acetone to 80% concentration yielded a relatively small precipitate which was inactive. Saturation with ammonium sulfate yielded a small precipitate without activity. Dialysis of the water solution of the 60% alcohol centrifugate against running tap water for 20 hours resulted in loss of activity of material remaining within the membrane. Results are detailed in Table II.

Discussion. The results presented establish a positive answer to the initial objective, that is, there is a factor which will counteract the growth inhibitor of raw soybeans. Although the results presented indicate that the factor occurs only in crude trypsin powder, preliminary data seem to indicate that the anti-SGI factor may have a more widespread occurrence but at insignificant levels when assayed against 25% raw soybean oil meal.

The feeding of crude trypsin powder was initially undertaken in an effort to remove the trypsin inhibitor from soybean extracts as the trypsin-trypsin inhibitor complex of Kunitz (5). As shown by the results, such addition of crude trypsin powder to the ration did counteract the growth inhibiting effect of raw soybeans. However, data presented indicate that addition of 5 parts crude trypsin powder to the ration containing 25 parts raw soybean oil meal reduced the trypsin inhibitor activity to approximately 80% of that of raw soybean alone. Also, since autoclaving destroyed trypsin activity but not the anti-SGI activity of the trypsin powder, it is evident that trypsin

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3. Borchers, R., Ackerson, C. W., and Sandstedt, R. M., *Arch. Biochem.*, 1947, v12, 367.

4. Anson, M. L., *J. Gen. Physiol.*, 1938, v22, 79.

5. Kunitz, M., *J. Gen. Physiol.*, 1947, v30, 311.

TABLE II. Some Properties of the Anti-SGI Factor in Trypsin Powder.

Trypsin powder $\Bumpeq$ 5% (see text for details)	Rat growth g/day ± SE g gain/g food consumed Soybean oil meal Autoclaved	 Raw	Raw autoclaved, %
Autoclaved	3.84 ± .24 (.50)	3.88 ± .18 (.44)	101
Autolysate soluble	3.37 ± .29 (.49)	3.44 ± .14 (.51)	102.1
pH 3, 60% ethanol soluble	3.40 ± .14 (.46)	3.46 ± .11 (.45)	101.7
n-butanol insoluble	3.39 ± .19 (.46)	3.43 ± .18 (.43)	101.2
acetone ppt.	3.83 ± .15 (.45)	2.31 ± .08 (.28)	60.3
ammonium sulfate ppt.	2.99 ± .08 (.40)	2.16 ± .14 (.26)	72.3
dialysis residue	3.94 ± .13 (.49)	3.43 ± .15 (.42)	87.1

activity is not essential to the anti-SGI activity.

The importance of the anti-SGI factor of crude trypsin powder in animal nutrition is difficult to outline at present. Nutritional effects have been ascribed to preparations from pancreas, particularly lipocaic. Also, no untoward effects, that is deficiency symptoms other than depressed growth, have been reported as a result of feeding raw soybean oil meal. The relation of the anti-SGI factor of crude trypsin powder to other nutritional factors as well as to the normal nutrition picture requires further investigation.

Summary. Growth of rats fed a ration containing 25% raw soybean oil meal was improved to the same rate of gain as rats fed autoclaved meal when 5% crude trypsin powder was added to each ration. The ability of the crude trypsin powder to counteract the soybean growth inhibitor (SGI) has established the existence of an anti-SGI. That the anti-SGI activity of crude trypsin powder was not dependent upon trypsin was established by heat stability.

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Differences in Cellular Pathogenicity of Two Immunologically Related Poliomyelitis Viruses as Revealed in Tissue Culture.* (18982)

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With the exception of antigenicity, biological differences within the group of poliomyelitis viruses have not been intensively investigated. This may be due in part to the fact that convenient technics are not available for such a study, as is the case with the influenza viruses. However, recent results from this laboratory (1) have shown that 2 immunologically related poliomyelitis strains, Lansing and Yale SK, differ in their ability to elicit antibodies after feeding to cynomolgus monkeys. The work of Enders, Weller, and Robbins(2,3) on the growth of poliomyelitis viruses in tissue culture has opened new avenues for exploration in this field. These workers have recently reported the capacity of Lansing and Brunhilde strains to cause cell injury and death in human embryonic tissues(4). We had found that the poliomyelitis viruses will grow in monkey testes *in vitro* (but not *in vivo*),

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* Aided by a grant from the National Foundation for Infantile Paralysis.

† Predoctoral Sheffield Fellow of Yale University.

1. Melnick, J. L., *J. Immunol.*, 1951, v67, 219.

2. Enders, J. F., Weller, T. H., and Robbins, F. C., *Science*, 1949, v109, 85.

3. Weller, T. H., Robbins, F. C., and Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 153.

4. Robbins, F. C., Enders, J. F., and Weller, T. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 370.

5. Syverton, J. T., Scherer, W. F., and Butorac, G., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 23.

as have Syverton(5) and Evans(6), using the suspended cell cultures as recommended by Enders(2).

The present report is concerned with the growth of poliomyelitis virus in roller tube cultures of monkey testes, and particularly with the description of a technic whereby in 4 days, it is possible to titrate the virus using degeneration of fibroblasts of monkey testes as the criterion of virus growth. We have used this technic to compare the relative cytopathogenic effects of the antigenically related Lansing and Yale SK (Y-SK) strains of poliomyelitis virus.

Materials and methods. Tissue cultures. Roller tube cultures were employed in these experiments. The technic, used with some modification, has been described in detail(7). Minced testes from immature rhesus *(Macaca mulatta)* monkeys, and, in a separate series of experiments, minced human embryonic skin-muscle, were used. The medium consisted of 9 parts of the "HS" mixture as described by Weller and Enders(8), which consists of 3 parts of Hanks salt solution and 1 part of Simm's ox serum ultrafiltrate, and 1 part of chick embryo extract prepared from 10-16-day-old embryos extracted with acetone according to the procedure of Margoliash(9). This extraction of chick embryo tissue was found to be necessary in order that fibroblasts with no granular lipid deposits are obtained during growth. In this way, any degeneration due specifically to the virus may be clearly seen. Fifty units of penicillin and 50 μg of streptomycin per ml of medium were added. 0.9 ml of this mixture was added to each culture tube containing 6 to 10 pieces of tissue. The nutrient medium was changed whenever a drop in pH made it necessary. The tissue was allowed to grow 5 to 7 days before addition of virus. At this time, fibroblastic proliferation is moderate. *Viruses.* Lansing and Y-SK strains of poliomyelitis virus were used as 10% mouse cord suspensions. The LD_{50} of Lansing was 3.5, and that of Y-SK 3.0. The Lansing strain had undergone 267 passages in mice, while the Y-SK only 11 passages. 0.1 ml of each virus was added to each of 4 culture tubes. Four culture tubes inoculated with 10% normal mouse cord served as controls. *Titrations.* Virus titrations were performed by adding serial tenfold dilutions of the suspensions to be titrated to growing tissues. Three to 4 roller tubes were used per virus dilution, and 0.1 ml was used as the inoculum. Mouse titrations of the same suspensions were done by intracerebral inoculation (0.03 ml) of adult albino Swiss mice. Eight mice were used per virus dilution and they were observed daily for 5 weeks. The Reed-Muench method was used to calculate the LD_{50}. *pH changes.* pH changes were followed by taking the average of the readings of the pH of the medium in the 4 roller tubes before the addition of fresh medium. The pH was determined by colorimetric comparison with standard buffer solutions, which had the same buffer and the same concentration of phenol red as the medium. The pH of the fresh medium varied from 7.5 to 7.7.

Experimental. In order to compare the cytopathogenic effect of Y-SK and Lansing viruses, the 2 strains were inoculated at the same time into different tubes of the same growing testicular tissue. Four days after virus inoculation, the roller tubes inoculated with Y-SK, when examined microscopically, showed a complete destruction of the testicular fibroblasts. Granulation and loss of structure were evident. In marked contrast, the tubes inoculated with Lansing showed only a slight tissue destruction. These observations suggested that a titration of Y-SK, but not of Lansing, might be possible in roller tubes. This proved to be so. Consequently, several dilutions of the fluid phase of Y-SK inoculated tubes, at various intervals following virus inoculation, were titrated both in tissue culture and in mice. At this time the medium was also renewed. The culture fluids of the Lansing inoculated tubes could be titrated only in mice. The results, typical of a number of experiments, are given in Table I.

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7. Feller, A. E., Enders, J. F., and Weller, T. H., *J. Exp. Med.*, 1940, v72, 367.

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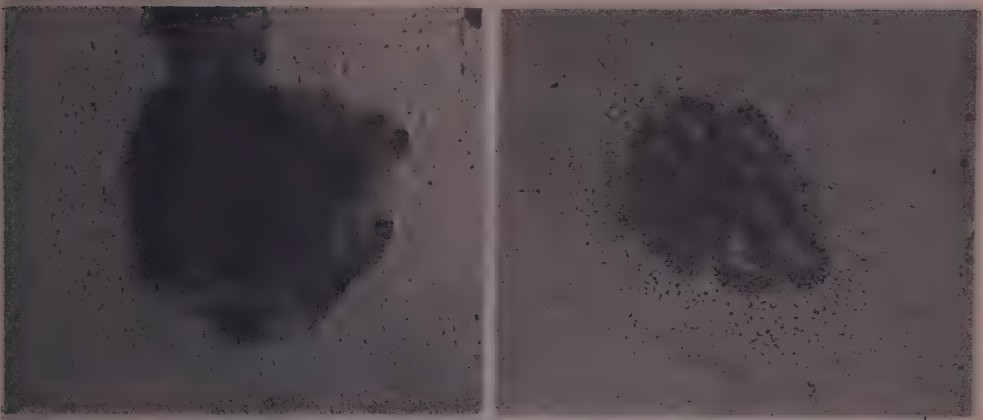
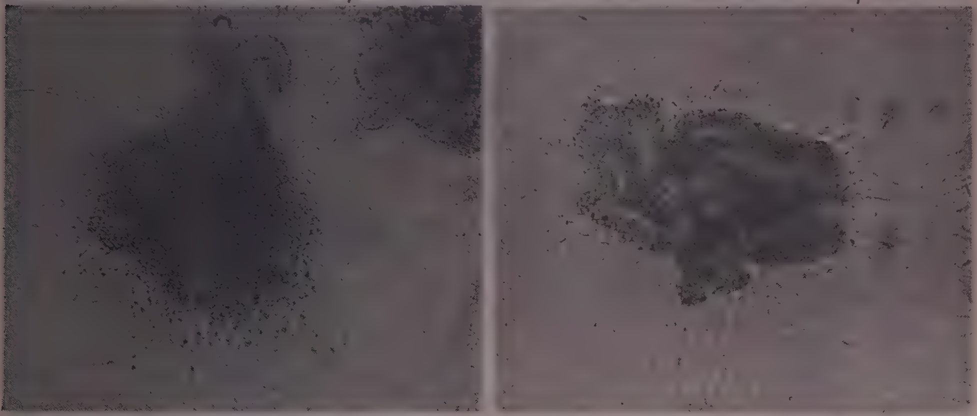


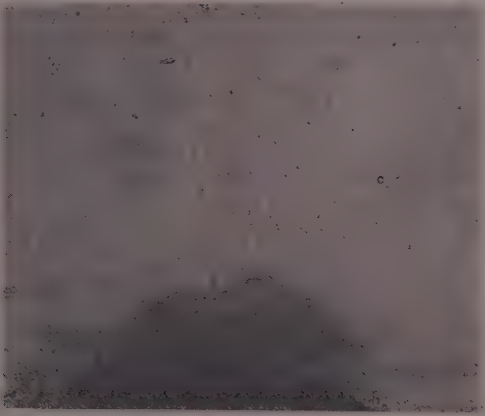
Tissue culture Y-SK virus, undiluted

Tissue culture Y-SK virus, 10^{-1}



Tissue culture Y-SK virus, 10^{-2}

Tissue culture Y-SK virus, 10^{-3}



Control tissue

FIG. 1. Titration of Y-SK strain of poliomyelitis virus in roller tube cultures of monkey testicular tissue. Magnification 67 ×.

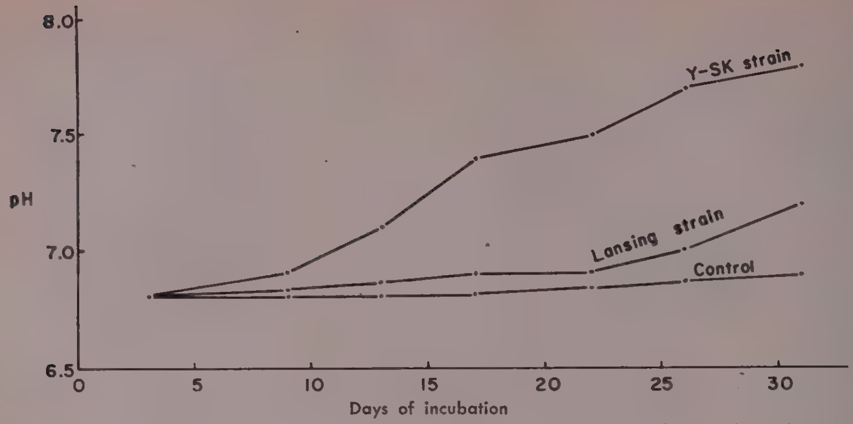


FIG. 2. pH determinations of fluid phase of roller tubes of monkey testicular tissue inoculated with Y-SK and Lansing poliomyelitis viruses.

These results indicate approximately equal susceptibility of monkey testicular tissue in roller tubes and the intact mouse to the Y-SK strain of poliomyelitis virus. This was substantiated by the fact that the tissue culture titration of the original inoculum gave an endpoint of 10^{-3}, the same as that found when the virus was titrated in mice. The fact that the Lansing strain grew equally as well as the Y-SK strain and still did not show as much degeneration points to a fundamental difference in the cellular pathogenicity of these two strains.

A typical tissue culture titration is illustrated in Fig. 1. The undiluted virus causes complete destruction of the fibroblasts, involving granulation, shrinkage, and loss of the normal structure shown by the control tissue. As the virus is serially diluted, correspondingly less fibroblastic degeneration is seen. The endpoint here is 10^{-2}, where definite degeneration of the tissue is still evident. In the tubes showing degeneration, the amount of tissue proliferation is less than that of the controls. The tissue inoculated with the 10^{-3} dilution of virus shows as much fibroblastic proliferation as the control tissue. Specific immune serum prevents tissue degeneration by the virus. Undiluted Lansing virus gives about the same amount of degeneration as the 10^{-2} dilution of Y-SK in Fig. 1.

Because of the marked tissue degeneration caused by the Y-SK strain of poliomyelitis virus in contrast to that caused by the Lansing strain, it might be expected that these two strains would also differ in their capacity to alter the metabolic activity of their host tissue. One manifestation of such an alteration would be a change in the amount of acid production corresponding to the amount of damage caused by the virus. Therefore pH changes induced by the two viruses were also followed throughout the experiment (Fig. 2). For the first 9 days of incubation, no significant pH change occurred in any of the roller tubes. Then from the 13th day until the termination of the experiment on the 31st day, the Y-SK inoculated roller tubes began to show a progressive decrease in acid production, with the pH rising from an initial value of 6.8 to 7.8. No such marked change occurred in the Lansing tubes, although the initial pH of 6.8 slowly rose to 7.2 by the 31st day of incubation.

To determine what the cytopathogenic effect of the Y-SK and Lansing strains used in the above experiments would be on another tissue, human embryonic skin-muscle roller tube cultures were tested. These experiments indicate that these two strains also differ in their cytopathogenic effect on this tissue. The Y-SK strain again caused a greater amount of fibroblastic degeneration in 4-6 days than the Lansing strain. The testing of various culture fluids, in a series of experiments similar to that described in Table I, revealed that the two viruses grew to an approximately equal

TABLE I. Tissue Culture and Mouse Titrations of Y-SK and Lansing Poliomyelitis Viruses in Roller Tube Cultures of Monkey Testicular Tissue.

	Negative logarithm of tissue culture fluid		
	Y-SK		Lansing
Days after inoculation	Tissue culture titration	Mouse titration, LD_{50}	Mouse titration, LD_{50}
3	1*	.8	1.2
6	2	1.8	1.2
9	2	1.5	1.6
13	2	1.2	1
17	2	1.1	.9
22	1	.4	.4
26	1	.2	.3
31	1	.6	.5

* The tissue culture endpoint is the final dilution of virus which produces tissue degeneration in 4 days, as shown in Fig. 1. In the series shown there, the end point was found in the 10^{-2} concentration of the culture fluid being titrated.

TABLE II. Mouse Titrations of Y-SK and Lansing Poliomyelitis Viruses in Roller Tube Cultures of Human Embryonic Skin-Muscle.

	Viruses	
	Y-SK	Lansing
Days after inoculation	Mouse titration (negative logarithm) LD_{50}	LD_{50}
3	*	*
6	1.4	1.7
9	1.2	1.6
12	1.1	1.6
15	1.1	1.5
19	1	1.1
23	*	*

* Not done.

extent in the human tissue. A representative experiment is given in Table II. Moreover, the pH changes in this experiment were similar to those obtained for the monkey testicular tissue (Fig. 3), in that the Lansing strain induced less of a pH change than Y-SK. After 23 days of incubation, the initial pH of 6.8 rose to 7.6 for the Y-SK strain, and to 7.2 for the Lansing strain.

Discussion. Viral variation is well established. Variation within an immunologically related virus group may involve such diverse properties, as pathogenicity, toxicity, and stability, as has been extensively studied in the case of the influenza group of viruses. It is reported here that the 2 immunologically related strains, Lansing and Y-SK, differ in their cytopathogenic effect on monkey testicular tissue, and human embryonic skin-muscle, as evidenced by a difference in the amount of tissue destruction, and in the alteration of the host metabolic activity. The fact that Y-SK produces a more marked tissue damage, raises

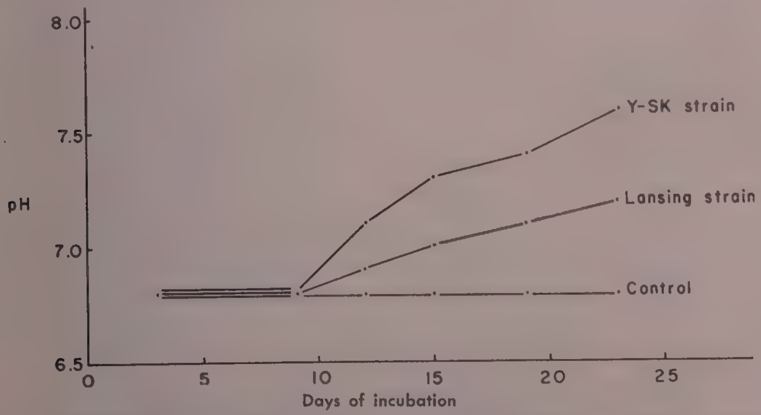


FIG. 3. pH determinations of fluid phase of roller tubes of human embryonic skin-muscle inoculated with Y-SK and Lansing poliomyelitis viruses.

the question whether this difference from the Lansing strain is due to the fewer number of mouse passages which the Y-SK has had, or to an inherent difference between the two strains. It is known that many laboratory virus variants have appeared as a result of continuous passage in a new animal host. Therefore the possibility exists that both viruses initially had the same cytopathogenic ability, and that for the Lansing strain, this ability was lost by continuous mouse passage. In this connection, Theiler(10) has found that continuous mouse passage of the Lansing strain resulted in a marked drop in its capacity to infect monkeys.

Summary. The Y-SK strain of poliomyelitis virus may be titrated in roller tube cultures by observation of the degeneration of monkey testicular fibroblasts within 4 days following the addition of virus to the tissue culture. The extent of fibroblastic degeneration is inversely related to the metabolic activity of the tissue, as measured by the amount of acid production. The 2 immunologically related Y-SK and Lansing strains of poliomyelitis virus differ in the degree to which they cause fibroblastic degeneration, and alter the amount of acid production by the tissue. In contrast to the Lansing strain, the Y-SK strain produces more extensive degeneration in fibroblasts of monkey testicles and also in those of human embryonic skin-muscle.

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Growth Promotion in Tetrahymena by Folic Acid Analogs.* (18983)

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(Introduced by C. A. Stuart.)
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The most widely used of the substituted folic acids in inhibition studies is 4-aminopteroylglutamic acid (Aminopterin). Early studies have shown it to be inhibitory to all organisms tested,† with the single exception of the rabbit, which had not been previously rendered folic acid deficient(1). It has been shown(2,3) that inhibition by low concentrations of inhibitor can be reversed by large doses of folic acid but reversal fails when the inhibitor concentration is high. More recently(4,5) reversal of inhibition has been obtained over wide ranges with citrovorum factor.

It is of considerable interest, therefore, to find that the animal microorganism, *Tetrahymena,* is not only uninhibited by Aminopterin but that this compound will replace folic acid in its diet. The same is true of N^{10}-methylfolic acid (Methopterin), although in this case the growth promoting power is lower and mild inhibition results at very high concentrations.

This report deals with Aminopterin, Methopterin, pteroylaspartic acid, 4-aminopteroylaspartic acid and citrovorum factor.

Experimental. The organism used was *Tetrahymena geleii* W, grown in bacteria-free culture. The medium used was Medium

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* Aided by a grant recommended by the Committee on Growth acting for the Am. Cancer Society and a grant from the Research Corporation.

† A note by G. E. Foley reports that the partial utilization (probably by deamination) of 4-aminopteroylglutamic acid by *Streptococcus faecalis* 8043 in appropriate pteroylglutamic acid-free substrate has been demonstrated (personal communication).

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TABLE I. Comparison of the Activities of Folic Acid, 4-Aminofolic Acid and N^{10}-Methylfolic Acid for *Tetrahymena.*

Compound	Amt (mγ/ml) required for half max growth No thymine	 40 γ/ml thymine	% activity No thymine	 Thymine
Folic acid	.131	.022	100	100
4-aminofolic acid	.76	.13	17.2	16.9
N^{10}-methylfolic acid	7	1.2	1.9	1.8

A(6) in which Tween 80 at 10 mg/ml was substituted for the Tween 85, and folic acid was omitted in appropriate experiments. All experiments were run in triplicate and the various tests were repeated a number of times. The procedure has been described elsewhere(7,8). Dose response tubes were inoculated with organisms which had been grown in the presence of only half maximal concentrations of folic acid for 7-8 days. The medium was autoclaved together with the folic acid or the derivatives (the Tween, dextrose and phosphate were autoclaved in a separate solution), after adjustment to pH 7.0 with the Beckman pH meter. All procedures were carried out in the virtual absence of white light, which is absolutely necessary when working with high dilutions of these light sensitive compounds. In the replacement experiments the results were checked with multiple serial transplants to insure against folic acid carry-over either in the medium or in the protoplasm of the organisms.

Results. It had earlier been shown that growth of *Tetrahymena* fails in the absence of folic acid(9-11) provided at least two serial transplants are made. Indefinitely transplantable growth occurs, however, in the presence of Aminopterin or Methopterin. The activity of Aminopterin is about 17%, while that of Methopterin is about 2% that of folic acid. The addition of thymine to the medium spares folic acid(12) and this compound spares both Aminopterin and Methopterin (Table I).

Aminopterin is capable of reversnig the inhibition caused by 4-amino-9-methyl-pteroylglutamic acid, 4-amino-N^{10}-methylpteroylglutamic acid and 4-amino-9,10-dimethylpteroylglutamic acid(13) when amounts possessing activity equivalent to folic acid are added.

The above results could be explained on the basis of folic acid contamination of the folic acid analogs. This is unlikely, however, because of the methods of synthesis.‡ Moreover, when the activities of commercial samples of both analogs were compared to the activities of highly purified samples§ they were found to be in good agreement. The likelihood of such close correspondence in degree of contamination between diverse samples seems remote.

It is impossible to say at the present time whether Aminopterin is deaminated and Methopterin demethylated to folic acid, or whether these compounds can function unchanged in place of folic acid. Some deamination of Aminopterin during heat sterilization appears to take place, as filter-sterilized material is somewhat less active. Autoclaving Methopterin, however, does not change its activity. It seems probable that *Tetrahy-*

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‡ Smith, J. M., Jr., private communication.

§ Obtained from Calco Chemical Division of American Cyanamid Company through the courtesy of Dr. J. M. Smith, Jr. The control number for the Aminopterin is R-2795-25-A and for Methopterin, R-2629-80.

mena possesses enzymes capable of changing both analogs to folic acid. Deamination, if it occurs, is not enhanced by increasing the concentration of pyridoxal in the medium, but this may mean that this specific deamination reaction is not mediated by way of a pyridoxal coenzyme. Moreover, the very low folic acid activity (less than 1%) of 4-dimethylaminopteroylgutamic acid[‖] might indicate that deamination is blocked by the methyl substitutions, and therefore, deamination is a step necessary for activity.

When equivalent activities of folic acid, Aminopterin and Methopterin are compared for their effect on the growth rate of *Tetrahymena* no differences are found. Slightly suboptimal amounts of any of these compounds causes growth rate depression, but maximum yields are finally attained.

Pteroylaspartic acid[¶] does not produce inhibition of the growth of *Tetrahymena,* but, unlike the other 2 analogs, it will not replace folic acid in the diet. Some folic acid sparing action occurs when this compound is added to medium containing suboptimal folic acid concentrations. Amino-an-fol[¶] (4-amino-pteroylaspartic acid) behaves similarly.

The first few samples of citrovorum factor (CF) which we obtained[‖] appeared to be more active in promoting growth of *Tetrahymena* than folic acid. This was due to the large amount of free folic acid present as unreacted starting material. Later samples of CF and of folinic acid[**] which were more highly purified were never greater in activity and usually less active than folic acid.

Discussion. Tetrahymena possesses an enzyme pattern unlike most other organisms in at least three distinct, though not necessarily unrelated, respects. First, it appears to be the only organism studied which has a requirement for folic acid together with an absolute requirement for purine(12,14). Second, the citrovorum factor is no more active than folic acid. Third, it appears unique in its ability ot utilize Aminopterin and Methopterin for growth.[†] According to Nichol and Welch(15) Aminopterin produces its inhibition in rats by interference with the conversion of folic acid to citrovorum factor. As CF does not appear to be the active form in *Tetrahymena* one should perhaps expect no inhibition. On the other hand, Dinning *et al.* (1) suggest that the site of action of Aminopterin is in its interference with choline oxidase activity. The recent report by Williams(16) that CF may be the prosthetic group of choline oxidase indicates that the previous observations may not be unrelated. It is possible that in *Tetrahymena,* as in the rabbit(1), choline oxidase is absent. Either of these hypotheses would explain the fact that Aminopterin is not inhibitory but would not explain its growth promoting activity. It would be interesting and important to determine whether or not Aminopterin would promote growth in a folic acid deficient rabbit. Law and Boyle(17) and Burchenal *et al.*(18) have shown that resistance to 4-amino derivatives of folic acid (including Aminopterin) develops in certain strains (L1210 and AK4) of mouse leukemia. It seems possible that these cells become resistant following the acquisition of enzymes capable of deaminating the 4-position, thereby reverting to a condition similar to what appears to be the case in *Tetrahymena.*

Summary. Tetrahymena geleii W is not inhibited by either 4-amino-pteroylglutamic acid or N^{10}-methylpteroylglutamic acid. Moreover, both of these analogs promote growth in the absence of folic acid. It is suggested that the organism possesses enzymes which deaminate the 4-position and enzymes

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[‖] Furnished through the courtesy of Dr. H. P. Broquist of the Lederle Laboratories.

[¶] Supplied by the Lederle Laboratories through the courtesy of Dr. T. H. Jukes.

[**] Furnished through the courtesy of Dr. F. R. Van Abeele of the Lilly Research Laboratories.

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which demethylate the 10-position. Pteroylaspartic acid and 4-amino-pteroylaspartic acid spare, but do not replace, folic acid. Citrovorum factor is never more active, and usually less active, than folic acid.

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Growth Inhibition in Tetrahymena by Folic Acid Analogs.* (18984)

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(Introduced by C. A. Stuart.)
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Tetrahymena geleii is at present the only organism known, with the exception of *Streptococcus faecalis* 8043(1), which can utilize Aminopterin, 4-amino-pteroylglutamic acid, in place of folic acid(1). This compound has proved to be an effective inhibitor of a variety of other organisms(2-5), but complete reversal of the effects of high concentrations can be obtained in such cases only by citrovorum factor(2,6-9), by folic acid and/or desoxyribosides(2,5,10,11). Methopterin, N^{10}-methylpteroylglutamic acid, was also found(1) to have some activity for *Tetrahymena* when used in place of folic acid. However, this compound proved to be slightly inhibitory when tested at sufficiently high concentrations.

The effect on the growth of *T. geleii* of other antifolics was, therefore, of interest. The present paper deals with the inhibition obtained with 4-amino-9-methylpteroylglutamic acid (A-ninopterin), 4-amino-10-methylpteroylglutamic acid (A-methopterin) and 4-amino-9,10-dimethylpteroylglutamic acid (A-denopterin).† These compounds have been shown to have an effect similar to that of Aminopterin in inhibiting mouse leukemias (10,11). A-methopterin reversibly inhibits the growth of *Streptococcus faecalis* R and irreversibly affects the growth of rats(12).

Material and methods. Tetrahymena geleii W was grown in Medium A(13) in which Tween 80 (10 mg/ml) was substituted for Tween 85. Preparation of the inoculum and all other conditions of growth have been previously described(1).

Results. Results with folic acid analogs were extremely variable quantitatively, although they were in good agreement qualitatively. Attempts to eliminate this variation by standardization of technics were made. Slight variations in the age and size of the inoculum made appreciable differences in quantitative responses. It was necessary, therefore, to repeat the experiments a large

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TABLE I. Comparison of the Inhibitory Effects of A-ninopterin, A-methopterin and A-denopterin.

Folic acid, γ/ml	A-ninopterin		A-methopterin		A-denopterin	
	γ/ml*	I.I.†	γ/ml*	I.I.†	γ/ml*	I.I.†
.0005	.545 ± .233‡ (3)§	1090	.209 ± .074‡ (2)§	418	.091 ± .008‡ (6)§	182
.001	.920 ± .240 (3)	920	.208 ± .057 (10)	208	.093 ± .014 (16)	93
.002	2.12 ± .52 (3)	1060	.223 ± .056 (16)	111	.138 ± .037 (20)	69
.004	3.69 ± .29 (3)	920	.255 ± .073 (17)	64	.177 ± .057 (17)	44
.005	4.82 ± .28 (3)	960				
.008			.298 ± .087 (19)	37	.217 ± .070 (13)	27
.01	8.65 ± 1.7 (4)	865				
.016			.324 ± .093 (14)	20	.278 ± .082 (11)	17
.02	16.6 ± 3.3 (3)	830				
.032			.393 ± .102 (12)	12	.450 ± .133 (12)	14
.05	42.2 ± 10.9 (3)	844				
.064			.554 ± .164 (8)	8.5	.648 ± .126 (4)	10
.10	75.5 ± 19.4 (3)	755				
.128			.699 ± .201 (2)	5.5	1.57 ± .017 (3)	12
.256					2.64 ± .032 (2)	10
.512					7.3 — (1)	14
1.024					36 — (1)	35
2.048			7.40 — (1)	3.6	88 — (1)	44
4.096			15.2 — (1)	3.7		
8.192			31.2 — (1)	3.8		

* Amount required for half max inhibition.

† Inhibition index defined as molar ratio between amount of metabolite present and amount of inhibitor required for half max inhibition at given concentration.

‡ Stand. dev.

§ No. in parentheses indicates No. of experiments.

number of times in order to obtain reliable averages. However, once these data had been obtained, the effects of the addition of other compounds could be studied with fewer repetitions. It was necessary merely to establish whether or not the results followed a similar pattern.

The 3 compounds tested fall into 2 categories. A-ninopterin behaved as a strictly competitive antagonist (Table I) over the entire range tested (0.0005-0.1 γ/ml). The inhibition index was nearly constant whether folic acid or citrovorum factor was used. On the other hand, both A-methopterin and A-denopterin have inhibition indices which decrease steadily as the concentration of folic acid is increased (Table I), until a fairly constant value is reached. Under certain circumstances it could be demonstrated that A-methopterin actually stimulates growth, when the concentration of folic acid was suboptimal and the concentration of A-methopterin was below the inhibitory level. This accounts for the fact that the amount required for half maximal inhibition at a level of 0.0005 γ/ml is the same or higher than that required at a level of 0.001 γ of folic acid /ml (Table I).

Citrovorum factor was used in place of folic acid in reversing the effects of these antifolic compounds. It was anticipated that the results would indicate a strictly competitive relationship, such as has been described in the case of Aminopterin(*2*). However, the data obtained were almost identical with those for folic acid. The citrovorum factor used was a synthetic product† and similar results were obtained with synthetic folinic acid.‡

The addition of various materials either known to participate or suspected of participating in folic acid metabolism has been shown to increase the effectiveness of folic acid in reversing the inhibition due to Aminopterin (*2,5,9,11*). It may be seen (Tables II and III) that the presence of vitamin B_{12}, thiamine, desoxyribonucleic acid (DNA), ribonucleic acid (RNA) or glycine did not have the effect of making the inhibition competitive with folic acid. In all cases the release was somewhat more effective in the presence of these compounds, although thymine, in particular, increased the inhibition at low levels

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TABLE II. Effect of Addition of Compounds Concerned in Folic Acid Metabolism Upon the Inhibition Produced by A-methopterin.

Folic acid, γ/ml	Additions: None	B$_{12}$ (.0001 γ/ml)	B$_{12}$ (.001 γ/ml)	Thymine (40 γ/ml)	DNA (500 γ/ml)	RNA (500 γ/ml)
.0005	.209* ± .074† (2)‡			.145* ± .035† (4)‡		
.001	.208 ± .057 (10)	.190* ± .010† (2)	.180* ± .000† (2)‡	.182 ± .053 (4)		
.002	.223 ± .056 (16)	.221 ± .004 (2)	.210 ± .012 (2)	.212 ± .055 (6)	.102* —† (1)‡	.231* ± .089† (4)‡
.004	.255 ± .073 (17)	.282 — (1)	.293 — (1)	.271 ± .068 (6)	.182 ± .044 (3)	.265 ± .099 (5)
.008	.298 ± .087 (19)	.340 — (1)	.340 — (1)	.331 ± .072 (12)	.222 ± .048 (3)	.346 ± .128 (5)
.016	.324 ± .093 (14)			.409 ± .083 (12)	.277 ± .062 (3)	.392 ± .150 (5)
.032	.393 ± .102 (12)			.495 ± .109 (5)	.326 ± .086 (3)	.502 ± .201 (5)
.064	.554 ± .164 (8)			.664 ± .169 (5)	.375 ± .005 (2)	.632 ± .316 (4)
.128	.699 ± .201 (2)			.957 ± .394 (3)		
.256				1.60 ± .73 (3)		
.512				2.20 — (1)		
1.024				4.35 — (1)		
2.048	7.40 — (1)			9.60 — (1)		
4.096	15.2 — (1)			22.6 — (1)		
8.192	31.2 — (1)			41.2 — (1)		

* Amt required for half max inhibition (γ/ml). † Stand. dev. ‡ No. in parentheses indicates No. of experiments.

TABLE III. Effect of the Addition of Compounds Concerned in Folic Acid Metabolism Upon the Inhibition Produced by A-denopterin.

Folic acid, γ/ml	Additions: None	B$_{12}$ (.0001 γ/ml)	B$_{12}$ (.001 γ/ml)	Thymine (40 γ/ml)	DNA (500 γ/ml)	RNA (500 γ/ml)	Glycine (100 γ/ml)
.0005	.091* ± .008† (6)‡			.076* ± .027† (2)‡	.064* ± .007† (2)‡	.215* —† (1)‡	
.001	.093 ± .014 (16)	.092* ± .017† (2)‡	.085* ± .021† (3)‡	.096 ± .034 (7)	.089 ± .006 (2)	.258 — (1)	.152* —† (1)‡
.002	.138 ± .037 (20)	.128 ± .033 (2)	.121 ± .031 (3)	.176 ± .070 (8)	.145 ± .040 (2)	.420 — (1)	.204 ± .057 (4)
.004	.177 ± .057 (17)	.168 ± .053 (2)	.153 ± .058 (3)	.217 ± .105 (6)	.238 ± .068 (2)		.292 ± .086 (3)
.008	.217 ± .070 (13)	.216 ± .079 (2)	.204 ± .073 (3)	.338 ± .154 (4)	.374 ± .106 (2)		
.016	.278 ± .082 (11)	.401 ± .119 (2)	.397 ± .077 (3)	.985 ± .325 (2)	.740 — (1)		
.032	.450 ± .133 (12)	.570 ± .120 (2)	.557 ± .100 (3)	1.66 ± .44 (2)			
.064	.648 ± .126 (4)						
.128	1.57 ± .017 (3)						

* Amt required for half max inhibition (γ/ml). † Stand dev. ‡ No. in parentheses indicates No. of experiments.

of folic acid.

It is of interest that fermentation folic acid and folic acid conjugate had exactly the same degree of effectiveness as folic acid on a molar basis.

Since Aminopterin can replace folic acid in the nutrition of *T. geleii,* it was used in an attempt to reverse the inhibition produced by these compounds. It was found to have the same efficiency in reversal as it has in growth promotion (18%).

Discussion. At the present time only conjectures can be offered in explanation of the results obtained with the antifolic compounds. It must be assumed that Aminopterin is either utilized as such or is deaminated to folic acid by *Tetrahymena*(1). There is no means of deciding at the moment which process occurs. However, it is more logical to assume that folic acid (or possibly the citrovorum factor) is the only growth-promoting compound in the group under consideration.

If it is true that *T. geleii* is capable of deaminating the 4-position of the pteridine ring, then it is possible that all of the analogs used in the present study are also deaminated. If that were the case, then A-methopterin would be converted to Methopterin, a compound which has growth-promoting activity in the concentrations used in these experiments. Thus, although only 0.0005 γ of folic acid/ml was added to the medium, the presence of a relatively large amount of deaminated A-methopterin would considerably increase the "folic acid activity". As the amount of folic acid in the medium increases, the contribution of the methyl folic acid would become relatively less important and would eventually become negligible. At this point one would expect the inhibition index to become constant or nearly so, as it does. The fact that A-methopterin is capable of sparing suboptimal amounts of folic acid is an indication that it can be converted to an active compound.

From the data at hand it is possible to calculate the amount of A-methopterin deaminated. In the expression $y = kx$, if y is the concentration of the metabolite and x is the concentration of the inhibitor required for half maximum inhibition, then k is the reciprocal of the inhibition index. In the case of A-methopterin, this index becomes constant at a value of about 4. It has previously been reported(1) that Methopterin can replace folic acid with an activity of about 2%. If, then, one lets z equal the amount of A-methopterin deaminated the following relationship would hold: $y + 0.02\ z = k\ (x–z)$. Substituting the values from Table I into this equation and solving for z, one obtains the values shown in Table IV. It may be seen that the values for z are reasonably constant. They might be expected to be somewhat lower at the lower concentrations of folic acid where growth is not quite optimal.

TABLE IV. Deamination of Folic Acid Analogs by Tetrahymena.

A-methopterin			A-denopterin		
x*	y†	z‡	x*	y†	z‡
.209	.0005	.192	.091	.0005	.086
.208	.001	.190	.093	.001	.083
.223	.002	.199	.138	.002	.118
.255	.004	.221	.177	.004	.137
.298	.008	.246	.217	.008	.137
.324	.016	.241	.278	.016	.118
.393	.032	.245	.450	.032	.130
.554	.064	.276			
.699	.128	.172			

* Amt (γ/ml) of inhibitor required for half max inhibition.

† Amt (γ/ml) of folic acid present.

‡ Calculated amt (γ/ml) deaminated.

The values for z in the case of A-denopterin (Table IV) were obtained in a somewhat different manner. Since nothing is known of the properties of the deamination product of A-denopterin, it is not possible to use the relationship given for A-methopterin. If one assumes that 9,10-dimethylpteroylglutamic acid neither promotes nor inhibits growth, then the equation becomes: $y = k\ (x–z)$. A value of $k = 1/10$ was used, since it gave the most constant values of z and is reasonably close to observed values. It may be seen however, that these values are not as constant as those obtained in the case of A-methopterin. This may be attributed to the fact that we do not know the properties of 9,10-dimethylpteroylglutamic acid in the metabolism of *Tetrahymena.*

The inhibition indices of A-methopterin and A-denopterin, when they reach constant values, are rather low, indicating that the compounds are very inhibitory. It is this fact that is responsible for the peculiar behavior of the inhibition indices of these compounds at intermediate concentrations of folic acid, since A-ninopterin, which is much less inhibitory, behaves as one would expect of a strictly competitive inhibitor. Since the amounts of A-ninopterin are relatively large, it is of little consequence whether or not small amounts are deaminated.

Summary. The antifolics, 4-amino-9-methylpteroylglutamic acid (A-ninopterin), 4-amino-10-methylpteroylglutamic acid (A-methopterin) and 4-amino-9,10-dimethylpteroylglutamic acid (A-denopterin) are all inhibitory to *Tetrahymena*. The inhibition produced by A-ninopterin is strictly competitive, being released over wide ranges of concentration of folic acid. The inhibition indices of the other two analogs decrease as the amount of folic acid in the medium increases until a certain level is reached, at which they become constant. This shifting index is tentatively explained on the basis of the ability of *Tetrahymena* to deaminate the 4-position of the analogs.

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Effect of Estrogen-Treatment and Castration on ACTH Content of Rat Adenohypophysis.* (18985)

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In a previous paper(1) we were able to show that the ACTH content of the rat's adenohypophysis is not affected by the severe depletion of acidophilic and beta-granular material which follows thyroidectomy. In the present study we sought to determine the effect of radical changes in the amount of the third described type of adenohypophysial secretory granulation, that of the delta cells, on the ACTH stores of the pituitary. For this purpose, the hypophyses of estrogen-treated and castrated male rats were assayed for their ACTH content against those of controls. Massive doses of estrogen were previously found to lead to a dramatic reduction in the number of delta cells(2), whereas castration is characterized by a progressive hypertrophy and hyperplasia of this cell type(3).

Method. The pituitaries of 46 young adult male Sprague-Dawley rats were used in this experiment. Twelve rats received daily injections of 62.5 μg α-estradiol in sesame oil over a period of 32 days, 10 rats were castrated and kept for 30 days and 24 intact animals served as controls. At the time of their sacrifice the estrogen-treated rats weighed 215-245 g (mean 226 g), the castrates 288-345 g (mean 313 g) and the controls 288-350 g (mean 328 g). All animals were killed by decapitation. The pituitaries of 4 castrates, 4 estrogen-treated and 6 intact rats were saved for quantitative histological examination. The remaining hypophyses were prepared for ACTH-assay by the method of Richards and Sayers(4). The simultaneous assay of the 3 groups of pooled donor pituitaries was carried out on hypophysectomized rats by the adrenal ascorbic acid depletion method of Sayers *et al.*(5). The recipients, the administered doses of the extracts, the procedure for the determination of the adrenal ascorbic acid concentration and the method for the statistical analysis of the assay data, as well as the

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TABLE I. Pituitaries of Estrogen-Treated and Castrated Rats Assayed for ACTH Content Against Those of Controls.

	Estrogen-treated donors (8)[*]		Control donors (18)		Castrated donors (6)	
Dose	No. of recipients	Response	No. of recipients	Response	No. of recipients	Response
1.25[†]	8	26.2[‡] ± 1.5[§]	10	27.2 ± 2.5	8	24.1 ± 1.9
.625	7	17.1 ± 2	8	19.6 ± 1.1	7	15.3 ± 2
.3125	7	8.8 ± 2	8	15.2 ± 2.2	9	7.7 ± 2.8
Total	22		26		24	
Slope of log dose-response curve		29.3		21.2		27.4
Significance of diff. between slopes			P = .2		P = .3	

For estrogen-treated vs. control $M = \bar{1}.8723$. $S_M = .06771$. For castrate vs. control $M = \bar{1}.7988$. $S_M = .07940$.

With that of controls at 100%, ACTH content of estrogen-pituitaries = 75%, range of potency at 95% level of confidence = 55-101%. ACTH content of castrate-pituitaries = 63%, range of potency at 95% level of confidence = 44-90%.

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[*] No. of donor rats.

[†] % donor pituitary per 100 g recipient.

[‡] Mean of differences between ascorbic acid concentration in left and right adrenal of individual recipients, expressed as % of concentration in left adrenal.

[§] Standard error.

TABLE II. Pituitary Weights and Cell Counts.

Group	No. rats	Wt, mg	Acidophil	Beta	Delta	Vacuolated delta
			%			
Control	6	7.2	45.10	1.84	8.50	.37
Castrate	4	9.5	40.05	2.55	17.85	.95
Estrogen-treated	4	7.5	52.62	1.48	.48	.00

histological staining technic applied to the pituitaries, were the same as in our previous study(1).

Results. The results of the assay are summarized in Table I and the pituitary cell counts in Table II. Since the histological findings on the castrate and estrogen-hypophyses are in good agreement with earlier observations on similarly treated rats(2,3), there is no reason to believe that they are not representative of the cellular composition of the assayed glands as well. It should be emphasized that the extremely low delta count of the estrogen-pituitaries still does not give an idea of the actual degree of delta-granule depletion from these hypophyses, as even the remaining few delta cells are reduced in size and poorly granulated. The elevated number of delta cells in the castrate pituitaries is a similarly poor measure of the plethora of delta granules in these glands, since the delta cells are not only hyperplastic in this condition, but also hypertrophic and loaded with granules.

The mean adrenal weight (per 100 g body weight) was 12.5 mg in the controls, 16.9 mg in the castrates, and 21.0 mg in the estrogen-injected group.

Discussion. Whereas previous observations failed to suggest any parallelism between delta cell count and adrenocorticotrophic function of the rat hypophysis(3), the present results indicate a similar lack of correlation between the amount of stored ACTH and delta granular material in the pituitary. The slight reduction in the ACTH content of the estrogen-pituitaries is by no means proportionate to the conspicuous degranulation of the delta cells and the enlarged castrate pituitaries, which contain an overabundance of delta granules, appear to have even lower ACTH stores. It seems justified to conclude that the delta granule can not be the cytological sign of ACTH storage.

High titers of pituitary ACTH having been found in coexistence with a large-scale depletion of the acidophilic, the beta(1), and, as the results of this paper show, the delta granulation, none of the 3 types of secretory granules in the hypophysis of the rat can be regarded as the morphological equivalent or even the site of ACTH storage. Since, on the other hand, the secretory granulation is the only reliable distinguishing mark between pituitary cell types, it is hard to conceive how the exclusive role of any given class of cells in the production and storage of ACTH could be convincingly established with our present histophysiological technics. Theoretically, two possibilities exist: (a) ACTH is produced by only one type of cell, or (b) it is elaborated by several or all classes of adenohypophysial cells, possibly including the chromophobes. In either case its storage does not appear to take place in the form of microscopic particles. The connection between ACTH and the uniform microsomes observed by Fernández-Morán and Luft(6) in basophil, acidophil and chromophobe cells of the rat pituitary remains to be explored.

The reduction in the amount of pituitary ACTH in both the castrates and the estrogen-treated rats of this study seems to have been due to an accelerated release rather than a block of formation, since the adrenals were found to be enlarged in both conditions. The lowered ACTH level in the castrate pituitaries might explain why Szego and White(7) observed a diminished adrenocorticotrophic response to the stress of starvation in castrated rats.

Emery and Winter(8), in contradiction to our observations, found no difference between the adrenal-stimulating potency of the pituitaries of castrated and intact male rats. As these authors did not use hypophysectomized recipients, the reliability of their assay method is open to serious doubt.

Summary. (1) The ACTH content of the pituitaries of estrogen-treated and castrated male rats was assayed against that of controls by the adrenal ascorbic acid-depletion method. (2) The estrogen-hypophyses were found to contain 75% and those of the castrates 63% of the amount of ACTH encountered in the controls. (3) Since no parallelism was observed between the ACTH content and the amount of delta granules present in these pituitaries, it is concluded that adenohypophysial ACTH is not associated with the delta granules. (4) Further histophysiological and physiological implications of these findings are discussed.

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Sinusoidal Dilatation in Liver and Adrenal Cortex of Tumor-Bearing Rats.*
(18986)

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The weights of the liver, spleen, adrenals and other organs of tumor-bearing rats and mice are reported as significantly increased over that of normals(1,2). In some instances this increase in total weight of the organ is associated with increased water con-

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FIG. 1. Liver, tumor-bearing rat No. 247 ROLV, showing small degree of sinusoidal dilatation. Tumor wt 107 g. Haematoxylin and eosin. ×-165.

tent, but to what extent the increase in weight can be accounted for by the presence of additional water or of dry residue is not clear in every case. Also the vastly dilated sinusoids of the liver and other organs in the case of tumor-bearing mice(1,3), suggests this as a possible site of the excess water in tumor-bearing rats.

In studies on rats bearing the Walker rat tumor 256, a series of 15 animals bearing tumors ranging in size from 10 g to 121 g was sacrificed by decapitation and the adrenals and liver removed and fixed in Bouin's solution or in 10% formalin. Controls were 5 animals that were uninoculated or in which the inoculum did not take. Paraffin sections of livers and adrenals were stained in hematoxylin and eosin or in iron hematoxylin. While no differences were found with respect to sinusoidal conditions in various regions of the liver, for purposes of comparison we used only sections taken from the anterior edge of the left lobe. Water content of the livers of tumor-bearing and control animals was determined from tissue samples by a method previously described(2), and found to average 73.3% and 69.3%, respectively. The adrenals were sectioned sagittally and sections through the middle of the gland saved for study.

On examining the livers and adrenals of these rats we were not able to find sinusoidal conditions that corresponded to those described for mice. In none of the livers of tumor-bearing rats could we find very marked morphological sinusoidal changes (Fig. 1). Measurements of sinusoidal diameters in 50 random fields of liver sections from an animal with a 121 g tumor gave an average value of 17.6 μ, while a similar count of a control gave 17.3 μ. With respect to the adrenal cortex, an essentially similar condition was found, except that dilatation of the sinusoids was somewhat more pronounced (Fig. 2). No detectable sinusoidal dilatation was seen in the spleen or kidney.

Although the increased water content of the liver may be determined when the transplanted tumor is as small as 10 g(2), there is no accompanying histological change. The slight sinusoidal changes that are occasionally seen, usually in rats bearing large tumors, are inconstant and of doubtful importance. It seems to us unlikely, on the basis of these

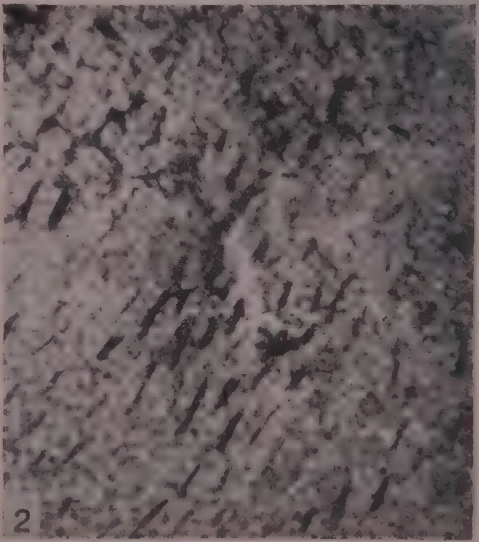

FIG. 2. Adrenal cortex, tumor-bearing rat No. 408 RV. The black areas are mildly dilated sinusoids occluded by erythrocytes. Tumor wt 121 g. Iron haematoxylin. × 195.

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3. Wolstenholme, J. L., and Gardner, W. U., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 659.

observations, that any significant amount of additional free water in the livers of tumor-bearing rats is present in the sinusoids. This must be sought, rather, in the cells, the intercellular spaces, and the blood. In the adrenal cortex, the sinusoidal dilatation is relatively greater than in the liver though hardly enough to assume this as the only site of a greatly increased water content for this organ.

Summary. The increased water content of the livers and adrenal glands of tumor-bearing rats is not correlated with an increase in caliber of liver or adrenal cortex sinusoids.

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In vitro Antibacterial Activity of Dimethylaminoethyl Substituted Compounds. (18987)

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In the course of systematic investigations* of alkyl-substituted dimethylaminoethyl ethers and dimethylaminoethyl amines we have surveyed their antimicrobial activity *in vitro*. The results of preliminary screening tests are presented below.

Experimental. Tests were performed against *Staph. aureus* 209, *B. mycoides* ATCC 6462, *Klebsiella pneumoniae* Type A ATCC 6450, *E. coli* ATCC R 5190, *Vibrio metchnikovi* and *Sh. sonnei* ATCC grown in Difco nutrient broth, *Diplococcus pneumoniae* A 5 grown in blood broth, *Cl. welchii* ATCC 460 grown in thioglycollate broth, and *Mycobacterium tuberculosis* H 37 RV and B 1, both grown in Dubos' liquid medium. The inoculum of non-acid fast organisms was 0.1 ml of a 1-500 dilution of an 18-hour culture. The inoculum of tubercle bacilli was 0.1 ml of a 14-day culture in Dubos' medium which had been diluted to an optical density of 0.2 as measured in Lumetron colorimeter. Final readings were taken after 3 days incubation with non-acid fast bacteria, and after 2 weeks incubation with tubercle bacilli. In addition, some of the compounds were assayed against *Trichophyton mentogrophytes*† grown in 4% dextrose, 1% peptone broth. The inoculum was 0.1 ml of a suspension, containing 15,000-25,000 spores, prepared from a 10-day culture on Sabouraud's dextrose agar. Cultures were incubated at room temperature (20-22°C), and final readings were taken after 15 days incubation. The compounds were dissolved in the medium used for each organism at a starting concentration of 64 mg % (w/v). A 2-fold serial dilution method was used throughout. Controls were included in every series and each test was run in duplicate. Some of the more active compounds were tested against a wider spectrum of pathogenic fungi, including *Trichophyton rubrum* ATCC 671, *Sporotrichum schenckii* ATCC 10213, *Sporotrichum floccosum* ATCC 10227, *Microsporum canis* ATCC 10214, *Microsporum audouini*, and *Candida albicans* ATCC 2091, using the method outlined above.

Results. Using the compound $R–O–CH_2–CH_2–N\ (CH_3)_2$, and replacing R in the aliphatic series, no activity was found against any organism until R = heptyl (8 mg% against *M. tuberculosis* B 1). When R = octyl slight activity appeared (64 mg %) which gradually increased as R increased to dodecyl (0.5-4 mg %). In general *V. metchnikovi* was slightly more sensitive to these compounds than were the other organisms tested. Of the cyclic ethers tested, B-pyridyl, phenyl, cyclohexyl, and benzyl were inactive, thymyl and methyl were weakly active (64 mg %), while tetrahydro furfural was moderately active (4-8 mg %).

Using the compound $R_1\ R_2\ N–CH_2–CH_2\ N\ (CH_3)_2$, and replacing R_1 and R_2 by aliphatic or cyclic groups, marked activity was found against all the fungi under test when R_1 = dodecyl, R_2 = methyl, and when R_1 = naphthyl, R_2 = hexyl, heptyl, octyl, nonyl, and decyl (.25-8 mg %). Low or moderate activity was found when R_1 = naphthyl, R_2 = methyl, ethyl, and propyl (4-64 mg %).

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* To be published from these laboratories.

† Obtained through the kindness of Dr. R. H. Benham, College of Physicians and Surgeons, New York City.

No activity was observed among the lowest members of the series of aliphatic dimethylaminoethylamines. Using the compound $\genfrac{}{}{0pt}{}{R_1}{R_2}> N\text{–}CH_2\text{–}CH_2\ N(CH_3)_2$, and replacing R_1 and R_2 in the aliphatic series, no activity appeared until $R_1 = R_2$ = butyl, when *M. tuberculosis* H37RV was inhibited by .5 mg %. The compounds $R_1 = R_2$ = methyl, $R_1 = R_2$ = ethyl, $R_1 = R_2$ = propyl, R_1 = allyl, R_2 = isopropenyl, R_1 = butyl, R_2 = ethyl, and R_1 = amyl, R_2 = methyl were all without effect on all organisms tested. When R_1 = amyl, R_2 = propyl, isopropenyl, or butyl, the compounds showed activity against *K. pneumoniae* and *Sh. sonnei* (.25 mg %) and weaker activity against gram positive organisms. When $R_1 = R_2$ = amyl antibacterial activity was low, as was the case when R_1 was hexyl, R_2 was methyl, ethyl, or propyl. In contrast R_1 hexyl, R_2 isopropyl inhibited *K. pneumoniae* and *Sh. sonnei* at .25 mg %. In ascending this series, when R_1 was amyl and R_2 was propyl, activity against gram negative organisms appeared. Bacteriostatic effects were maintained until R_1 was hexyl and R_2 was allyl. Activity then diminished, to reappear with a broader spectrum when R_1 was octyl and R_2 was hexyl. Maximal potency against both gram negative and gram positive organisms was obtained when R_1 was hendecyl or above. The most active members of this series of compounds were also effective against *Trichophyton mentagraphytes*. Optimal activity appears to be associated with a long chain hydrocarbon radical, but any such generalization would exclude the hexyl allyl derivative and would not account for the effectiveness of the lower members of the series against gram negative organisms.

Discussion. The more active members of the series of substituted dimethylaminoethylamines inhibited the growth of 4 g positive organisms, including aerobic and anaerobic spore formers, 4 g negative bacteria, and 2 varieties of tubercle bacilli. In addition, they were found to be active against a representative spectrum of pathogenic fungi. Although tests for bacteriostatic effects give no indication of germicidal activity, the above data indicate which of the compounds should be studied further. Work designed to evaluate the therapeutic potentialities of these compounds will be reported separately.

Conclusions. (1) No appreciable antimicrobial activity was found in an *in vitro* examination of a series of dimethylaminoethyl ethers. (2) Significant activity was observed in various long-chain di-aliphatic dimethylaminoethylamines. (3) When one of the aliphatic substituents was replaced by a naphthylene group marked antimicrobial activity was found.

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Thermolabile Antigens of *Shigella boydii* 2 Cultures with Special Reference to an Encapsulated Culture. (18988)

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The purpose of this paper is to report the natural occurrence of an encapsulated culture of *Shigella boydii* 2 (P288) and to describe the relationships of the thermolabile substance of the capsule to thermolabile somatic antigen in other *S. boydii* 2 strains and in *Klebsiella* and *Escherichia* cultures.

Fletcher(1) reported the occurrence of

mucoid variants in paratyphoid and dysentery bacteria but did not describe capsules in the dysentery bacteria nor the nature of the mucoid material. Various investigators reported that some strains of *Shigella dysenteriae* 1 (Shiga bacillus) are inagglutinable and Bauch(2) and Schuetze(3) noted that such cultures were made more readily agglutinable after they were heated at 100°C for ½ hour. Archer(4) described a thermolabile factor in Alkalescens-Dispar 01 *(Shigella alkalescens)* cultures which inhibited O agglutination but did not determine the nature of the thermolabile substance. Barnes(5) mentioned that inagglutinable cultures of *Shigella dysenteriae 2 (Shigella ambigua)*, *Shigella flexneri* 6, *S. boydii* 2, and *Shigella boydii* 5 (P143) had been encountered. However, a search of the literature failed to reveal any previous description of the occurrence of encapsulated shigellae. Kauffmann(6) described thermolabile envelope antigens in coliform bacteria which later were called L antigens. Envelope antigens of the L variety were encountered in *Escherichia coli* cultures that belonged to various O antigen groups. Kauffmann(7) reported that the thermolabile substance in Alkalescens-Dispar (A-D) cultures that inhibits O agglutination is an L antigen which occurs in certain *E. coli* cultures. Also, the relationship of the L antigen of A-D and *E. coli* cultures was mentioned by Frantzen(8). Kauffmann(9) reported that some *Escherichia* cultures contained an antigen that differed from L antigen in certain characteristics. Knipschildt(10) studied this antigen further, found that it was a relatively thermostable capsular substance, and designated it as A antigen. A third variety of envelope antigen, designated B was described by Knipschildt (11). Subsequently, these three thermolabile antigens were grouped together under the designation K (Kauffmann)(12). All 3 varieties, L, A, and B, are envelope antigens which inhibit O agglutination but the 3 differ in respect to the degree of their thermolability, antibody binding power after heating, and certain other characteristics(12,13). Utilizing the methods for determination of K antigens advised by Kauffmann, Madsen(14) studied *Shigella flexneri* cultures and reported that some *S. flexneri* 6 cultures contain a thermolabile antigen similar in its behavior to that of the B antigen of Knipschildt(11). *S. boydii* 2 is not one of the common *Shigella* types. It was described first from the United States by Morris, *et al.*(15). Twelve cultures of the type were employed in the present investigation. These included a type culture (P288, Poona), received from Brigadier J. S. K. Boyd in 1944; a culture isolated in Italy in 1944; one from Dr. W. W. Ferguson, and 9 cultures that were sent to this laboratory for identification between July, 1948 and May, 1951. These 9 cultures were isolated in Arizona, California, and New Mexico.

The biochemical reactions of the 12 *S. boydii* 2 cultures are as follows: Glucose and mannitol are utilized without gas formation within 24 hours incubation and maltose is fermented after 9 or 10 days incubation; lactose, sucrose, salicin, adonitol, and dulcitol are not utilized within a 30-day period; the bacteria are nonmotile, do not produce acetylmethylcarbinol, do not grow on Simmons' citrate agar nor hydrolyse urea, do not utilize

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12. Kauffmann, F., *J. Immunol.*, 1947, v57, 71.
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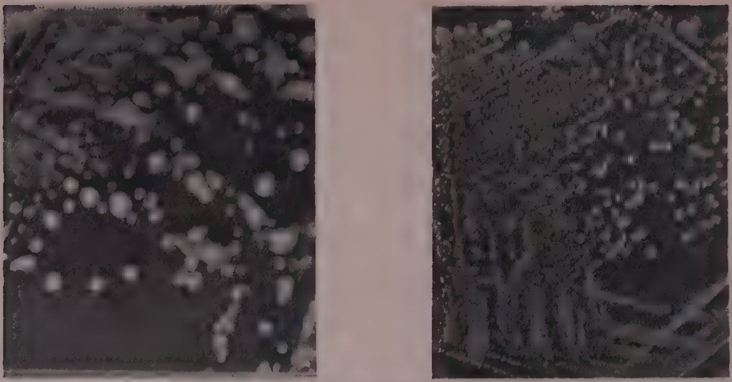

FIG. 1. Colonies of encapsulated and non-encapsulated *S. boydii* 2 cultures. Left, colonies of encapsulated culture. Right, colonies of non-encapsulated culture (reduced 1/10).

citrate in the medium of Christensen(16), and do not produce indol; all cultures are methyl red positive and reduce nitrates to nitrites.

The methods employed for the determination of O and K antigens were those advised by Kauffmann(12,13) for study of these antigens in *Escherichia* and *Klebsiella* cultures. Antiserums that were used for determination of the heat stable somatic antigens of the bacteria were prepared from 18-hour broth cultures which were heated for 2.5 hours at 100°C. Antiserums for the K, or thermolabile somatic, antigens were prepared by injection of unheated formalinized broth cultures that were incubated for 4 hours at 37°C. Agglutination tests for K antigens were made with formalinized (0.5%) 4-hour broth cultures and incubation was at 37°C for 2 hours followed by overnight incubation at 4 to 5°C. Tests for O agglutination were made with 18-hour broth cultures which were heated for 1 hour at 100°C and the tests were read after overnight incubation in a 52°C water bath. Examinations for capsules and for quellung reactions were made with living 4-hour broth cultures. The presence of capsules was determined in moist mounts with Pelican India ink. Living cultures of 5 of the 9 more recently isolated *S. boydii* 2 strains were inagglutinable in O antiserum when received. All cultures were agglutinated to the titer of O antiserum for *S. boydii* 2 when heated suspensions were used as antigens. One of these cultures, 4158/50, was mucoid and upon examination of a young broth culture was found to be encapsulated. The capsules were as large as those usually seen in *Klebsiella* cultures. Plating of this culture on infusion agar resulted in the appearance of 2 colony forms (Fig. 1). The first was an opaque, glistening, mucoid colony, and the second a translucent, smooth, and somewhat less raised colony form. Living cultures, made from the translucent O colony forms, were readily agglutinable in O antiserum while living cultures from the opaque, or K, colony forms were inagglutinable. The other inagglutinable cultures of *S. boydii* 2 also gave rise to 2 colony variants upon plating but the opaque, inagglutinable K forms were not mucoid or encapsulated. Upon plating, all of the cultures gave rise to these 2 colony forms, but in the case of those cultures that were agglutinable in the living state when received, K forms were few in number. However, by plating and selection, K colony forms were derived from all of the *S. boydii* 2 cultures including the older stock cultures.

Heated suspensions and O colony variants of the 12 *S. boydii* 2 cultures were agglutinated to the titer of an O antiserum for the type. Furthermore, heated suspensions of the encapsulated culture, 4158/50, removed all agglutinin from a *S. boydii* 2 antiserum. Reciprocally, all O agglutinin for culture 4158/50 was removed from an antiserum pre-

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16. Christensen, W. B., *Res. Bull. Weld. Co., Colo. Health Dept.*, 1949, v1, 3.

TABLE I. Quellung Reactions of Encapsulated *S. boydii* 2 Culture and *Klebsiella* Types.

	Antiserums		
Suspensions*	*S. boydii* 2 K antiserum	*Klebsiella*, Type 21	*Klebsiella*, Type 11
S. boydii 2 (4158/50, encapsulated)	16†	4	1
Klebsiella, Type 21 (1702/49)	4	32	2
Klebsiella, Type 11 (390)	1	4	16

* Broth cultures incubated 4 hr at 37°C then formalinized.

† Figures represent reciprocal of highest dilution that gave positive reaction.

pared with that culture when aliquot portions of the antiserum were absorbed with each of the 11 other *S. boydii* 2 cultures. Thus, it is clear that the O antigens of culture 4158/50 and the other *S. boydii* 2 strains are identical as demonstrated by reciprocal absorption tests. An antiserum prepared with a young living broth suspension of the encapsulated culture, 4158/50, reacted in a dilution of 1:320 with a living broth suspension of the homologous culture. The type of agglutination that occurred with typical K agglutination, firm discs were formed similar to those seen in capsular agglutination of other bacteria. This antiserum gave a positive quellung reaction in a dilution of 1:16 with the encapsulated form of the homologous culture (Table I). The homologous O agglutinin titer of this antiserum was 1:2560 to 1:5120 and heated suspensions of all 12 *S. boydii* 2 strains reacted in it to titer.

By selection of opaque colony forms, it was possible to demonstrate the presence of K antigen in all of the *S. boydii* 2 cultures. The 12 cultures gave K agglutination in dilution of 1:40 to 1:320 in K antiserum prepared with culture 4158/50. These same antigens were tested in *S. boydii* 2 O antiserum to determine the extent of inhibition of O agglutination. O agglutination was either partially or completely inhibited in all cases. Culture 4158/50 did not react even in a dilution of 1:40, while the other cultures were agglutinated partially in dilutions of 1:80, 1:160 and 1:320 in an antiserum the O titer of which was 1:10,240. The results are summarized in Table II.

Several attempts were made to prepare a pure K antiserum by absorption of the KO antiserum prepared with encapsulated culture 4158/50. This antiserum was absorbed with suspensions of the homologous culture and with several other *S. boydii* 2 cultures which were heated for 2.5 hours at 100°C and washed twice by centrifugation. In each instance all agglutinin for both O and K antigens was removed from the antiserum. Also, attempts were made to prepare a pure K antiserum by absorption of antiserum with minus forms (O forms) of the homologous and other *S. boydii* 2 cultures. These attempts failed because it appears that the absorbing suspensions still contained some K antigen and thus the colonies were not true minus forms. The results of these experiments indicate that the K antigen of culture 4158/50 is probably a B antigen because its antibody binding power was not destroyed by heating the suspensions at 100°C.

The results of direct K agglutination tests indicated that the 11 other *S. boydii* 2 cultures contained a K antigen related to that found in the encapsulated 4158/50. In order to determine whether or not the K antigen was the same in all strains examined, aliquot portions of antiserum prepared from the capsulated form of 4158/50 were absorbed with living suspensions of each of the 11 other *S. boydii* 2 cultures. In all cases, the K agglutinin was removed from the antiserum and the antiserum no longer gave a quellung reaction. This indicated that the K antigen was the same in all of the cultures examined. However, it is clear that there is considerable difference in the amount of K antigen present in the various cultures. Culture 4158/50 contains the greatest amount of K antigen as manifested by complete inhibition of agglutination of living suspensions in O antiserum and by the presence of capsules. It can be argued that the presence of a visible capsule on culture 4158/50 is not due to the production of a larger amount of K antigen, but simply due to the fact that it accumulated on the cells of 4158/50 but not on the cells of the other cultures. If this were true, a larger amount of K antigen should be found in the supernatant fluid of broth cultures of the

TABLE II. Serological Reactions of *S. boydii* 2 Cultures.

	S. boydii 2 antiserums			
	O antiserum		KO antiserum	
S. boydii 2 cultures	Living antigen	Heated antigen (1 hr 100°C)	Living	100°
4158/50 (encapsulated)	0*	10,240	320	2,560
66 (representative of non-encapsulated strains)	160†	10,240	160	5,120

* O equals no agglutination in dilution of 1:20 or higher.
† Figures represent reciprocal of highest dilution in which agglutination occurred.

non-encapsulated strains. Such is not the case. The supernatant fluid of culture 4158/50 produced more voluminous disc precipitates in precipitin tests and was active in much higher dilution than the supernatant fluids of the remaining 11 cultures. It was necessary continually to select moist, opaque colonies if culture 4158/50 were to be maintained in the encapsulated state. Serial transfer on the usual stock mediums resulted in a rapid disappearance of the capsule.

The relationship of the K antigen of *S. boydii* 2 culture 4158/50 to those of *Klebsiella* and *Escherichia* types was also investigated. The capsular material of culture 4158/50 was found to be related closely to the capsular antigen of Klebsiella type 21 (1720/49) and in a lesser degree to *Klebsiella* type 11 (390) (Table I). No reaction was obtained in other *Klebsiella* antiserums. The possibility of the coexistence of an O antigen relationship between the two *Klebsiella* types and *S. boydii* 2 was investigated, but it was found that only insignificant minor O antigen relationships existed. Living suspensions of 59 *Escherichia coli* cultures, containing the known 59 K antigens of that group, were tested in the KO antiserum for culture 4158/50. No relationship was noted between the K antigen of culture 4158/50 and any of the 59 K antigens of the *E. coli* group.

Comment. The writers found no other report in the literature of the presence of capsules in bacteria of the genus *Shigella*. It is clear, however, that occasional encapsulated *Shigella* cultures do occur and that living cultures of encapsulated strains are inagglutinable in O antiserums. Also, the data given above indicate that other inagglutinable *S. boydii* 2 cultures possess a similar antigen though they may not possess enough of the antigen to manifest itself in the form of a capsule. Studies are in progress to determine whether or not the thermolabile somatic antigens of other *Shigella* types may appear as capsules. An encapsulated culture of *Shigella boydii* 1 (Boyd 170) has been found. Living suspensions of this culture are inagglutinable, while heated suspensions are agglutinated to the titer of *S. boydii* 1 antiserum. The capsular antigens of *S. boydii* 1 and *S. boydii* 2 are not related.

Extragenetic relationships of the capsular antigens of members of different groups of Enterobacteriaceae is not a new finding. Kauffmann(17) described mucoid (M) forms of certain *Salmonella* types, notably *Salmonella paratyphi* B. The mucoid antigen inhibited O agglutination and gave typical capsular agglutination in antiserum prepared with living cultures of the M forms. Perch(18) demonstrated relationship between the M antigen of *S. paratyphi* B and *Klebsiella* type 13. Recently Edwards (unpublished data) demonstrated that the M antigen of an encapsulated culture of *Salmonella bovis-morbificans* is also related to the capsular material of *Klebsiella* type 13. The capsular antigen of *S. boydii* 2 is not related to that of *Klebsiella* type 13, but is related closely to that of *Klebsiella* type 21.

Summary. 1. Encapsulated cultures of *Shigella* types are described, apparently for the first time. 2. An encapsulated culture of *Shigella boydii* 2 is described. The occurrence of the same K antigen in other non-encap-

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18. Perch, B., *Acta Path. et Microbiol. Scand.*, 1950, v27, 565.

sulated *S. boydii* 2 cultures is reported. An encapsulated culture of *Shigella boydii* 1 also is mentioned. The capsular antigens of *S. boydii* 1 and *S. boydii* 2 are not related. 3. The antigenic relationship of the capsular antigen of *S. boydii* 2 and the capsular antigens of *Klebsiella* types 11 and 21 is reported. This relationship was demonstrated by both agglutination and quellung reactions. No relationship was found between the K antigen of *S. boydii* 2 and any of the 59 K antigens of the *Escherichia* coli group.

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Effect of Cortisone, Related Hormones, and Adrenalectomy on Susceptibility of Mice to Virus Infections.* (18989)

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Cortisone treatment may affect the course of virus infection to the disadvantage of the host. This has been demonstrated for influenza A, influenza B, and mumps viruses in chick embryos(1); for influenza A virus in mice(2); for strain MEF 1 poliomyelitis virus in mice and hamsters(3); for a Coxsackie virus in adult mice(4), and for vaccinia intradermally in the guinea pig(5).

The present work demonstrates that cortisone increases the susceptibility of mice to certain neurotropic viruses, and further investigates the nature of this effect by parallel studies with other steroid hormones, adrenocorticotropic hormone (ACTH), and adrenalectomy.

Methods. Mice. Banks (Swiss) mice weighing 18 to 24 g (6 to 10 weeks old) were used, except for experiment III-12 in which Rockefeller Institute mice of similar age were used. Female mice were used throughout except that males were used for the testosterone treated groups in Exp. IV-27. Adrenalectomies were done about a week before virus inoculation, using ether anesthesia and a dorsal approach. Adrenalectomized mice were given isotonic saline instead of water to drink, but received no other treatment. Autopsies on 38 of the adrenalectomized mice revealed small fragments of adrenal tissue in 11, indicating that about 70% of the mice had been completely adrenalectomized. *Viruses.* All viruses were obtained from Dr. Koprowski of Lederle Laboratories, and were carried in this laboratory by serial intracerebral passage in mice. In the experimental mice all virus inoculations were intraperitoneal using a volume of 1.0 ml of serial 10-fold dilutions of virus-infected mouse brain suspension. Five mice were inoculated with each dilution. Routine virus titrations were performed by intracranial inoculation of Banks mice with 0.03 ml of 10-fold serial dilutions of the material being tested, usually using 3 mice per dilution. Titers, either intraperitoneal or intracranial, are expressed as the LD_{50} dilution using the above volumes. In calculating titers the Reed-Muench formula(6) was used. In Exp. VI-5 the 10^{-1} virus dilution killed only one of the 5 control mice, so in order to calculate an

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† This work was carried out during tenure of a Damon Runyon Clinical Cancer Research Fellowship.

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TABLE I. Effect of Cortisone on Susceptibility of Mice to Virus Infections.

		Cortisone dosage				Virus titers		
						LD_{50} dilutions		
Virus	Exp. No.	mg per dose	No. doses	Duration (days)	No. doses before virus	Controls	Treated	Log difference
West Nile	XII-11	1	5	5	3	$10^{-1.8}$	$10^{-2.5}$	.7
	VI-5	1	5	5	2	$10^{-0.6}$	$10^{-0.8}$	.2
	II-27	2.5	7	10	7	$10^{-1.7}$	$10^{-4.7}$	3
	III-12	2.5	5	5	3	$10^{-2.9}$	$10^{-5.7}$	2.8
	IV-27	2.5	6	9	5	$10^{-1.7}$	$10^{-2.8}$	1.1
	VI-5	2.5	5	5	2	$10^{-0.6}$	$10^{-2.5}$	1.9
	VI-5	10	1	1	1	$10^{-0.6}$	$10^{-1.8}$	1.2
Ilheus	I-10	2	9	12	6	$10^{-2.8}$	$10^{-5.2}$	2.4
	II-27	2.5	7	10	7	$10^{-3.3}$	$>10^{-6.0}$	>2.7
	II-27	5	1	1	1	$10^{-3.3}$	$10^{-5.8}$	2.5
Bunyamwera	II-27	2.5	7	10	7	$10^{-2.7}$	$10^{-5.0}$	2.3
	IV-27	2.5	6	9	5	$10^{-4.2}$	$10^{-5.2}$	1
Anopheles B	II-27	2.5	7	10	7	no deaths at 10^{-1}		0

TABLE II. Effect of ACTH on Susceptibility of Mice to West Nile Virus Infection.

	ACTH dosage					Virus titers		
						LD_{50} dilutions		
Exp. No.	mg	Doses	Schedule	Vehicle	No. doses before virus	Controls	Treated	Log difference
III-12	.5	20	q6h	saline	10	$10^{-2.9}$	$10^{-3.5}$	.6
III-12	2.5	4	q6h	,,	2	$10^{-2.9}$	$10^{-2.9}$	0
VI-5	4	10	q12h	gelatin	6	$10^{-0.6}$	$10^{-5.0}$	4.4

TABLE III. Effects of Adrenalectomy, and Steroids Other Than Cortisone on the Course of Virus Infections.

						Log difference (treated minus controls)	
Treatment	Exp. No.	mg per dose	No. doses	Duration (days)	No. doses before virus	West Nile	Bunyamwera
Desoxycorticosterone	IV-27	10	5	9	3	.6	−1.2
Testosterone	IV-27	25	4	9	3	.5	− .6
Estradiol	IV-27	2.5	5	9	3	.5	− .2
Proluton	IV-27	25	5	9	3	1.3	.8
Adrenalectomy	VI-5	—	—	—	—	.4	—

LD_{50} titer it was assumed that a theoretical 10^{-0} dilution would have been an LD_{100}. Mice dying less than 24 hours after virus inoculation were discarded from the group. All other mice dying within the 14 days following virus inoculation were considered as dying because of virus infection. Confirmation of virus infection as cause of death was obtained in most instances by observation of paralysis for 1 to 3 days before death, or by examination of brains of dead mice for presence of virus (by intracerebral inoculation of serial dilutions into other mice with resulting typical virus death pattern). *Drugs.* All drugs were clinical type preparations. Dosage schedules are indicated in the tables. Cortisone was given intraperitoneally daily for 5 or more days. When more than 5 doses were used, no treatment was given on weekends. Virus was given at least 36 hours after the start of drug treatments. In 2 experiments a single intraperitoneal dose of cortisone was used, followed by virus 4 hours later. Desoxycorticosterone acetate, testosterone propionate, progesterone, and estradiol dipropionate were administered subcutaneously daily.‡ The doses used (see tables) were chosen as the highest practicable dosage that did not cause death of the mice. ACTH‡ was given subcutaneously. When administered in saline it was given every 6 hours, and when administered in 30% gelatin (to prolong absorption) it was given every 12 hours. Saline injections were given to the untreated control mice in Exp. II-27, using the same volume, route, and frequency of injections as in the cortisone treated mice. In all other experiments the controls received no injections.

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‡ The authors are greatly indebted to the Schering Corporation for a gift of all steroids except cortisone used in these studies, to the Research Division of Armour and Co. for a gift of ACTH and the gelatin vehicle in which it was administered, and to Merck and Co. for cortisone.

In order to determine whether the dosage of cortisone used in most of these experiments (2.5 mg i.p. daily) was pharmacologically effective, spleen and adrenal weights were determined on 9 mice 24 hours after the 3rd, 4th, or 7th doses. Spleens represented 0.25% of total body weights in treated mice and 0.47% in 9 control mice. Adrenal weights showed no consistent difference between treated and untreated groups. All 3 dosage schedules of ACTH (Table II) also caused an average shrinkage of spleens to 50% of normal weight. The 5-day ACTH treatment schedules caused adrenal hypertrophy (adrenals of the 6 mice studied represented 0.058% of body weight in contrast to 0.036% in the 9 controls). The one-day ACTH treatment caused no adrenal hypertrophy when measured 6 hours after the last injection.

Results. Cortisone. Treatment with 2.0 or 2.5 mg of cortisone daily for 5 or more days rendered mice consistently more susceptible to lethal infection by the intraperitoneal route by West Nile, Ilheus, and Bunyamwera viruses. The increase in susceptibility varied from 10-fold to 1000-fold as measured by the LD_{50} dilution of virus. The data from all experiments with cortisone are recorded in Table I. In addition to the decrease in minimum amount of virus causing death, the effect of cortisone usually was also indicated by earlier deaths in the cortisone treated mice as compared with control mice receiving the same dose of virus.

Cortisone at a dose of 1 mg daily failed to show any effect on susceptibility to West Nile virus in 2 experiments. Two experiments were done with a single large dose of cortisone. Mice treated with a single 10 mg dose of cortisone 4 hours before West Nile virus showed a slight increase in sensitivity, and a single dose of 5 mg of cortisone caused a significant increase in sensitivity to Ilheus virus.

A single experiment was performed with Anopheles B virus, which is infective for normal mice only if administered intracerebrally, to see if cortisone-treated mice would be susceptible to intraperitoneally-administered virus. No increase in susceptibility to this virus was observed, although simultaneous studies showed that cortisone-treated mice were much more susceptible to infection by each of the 3 above mentioned viruses.

In order to see if cortisone had any effect on a virus *in vitro*, a West Nile virus preparation was mixed with cortisone and isotonic saline so as to give a final virus dilution of 10^{-1} and cortisone concentrations of 10, 1, and 0.1 mg/ml. These mixtures and a control virus preparation containing no cortisone were incubated for 2 hours at 37°C and then titered intraperitoneally and intracerebrally in mice. The results showed no difference in virus titer after exposure to 1 or 0.1 mg/ml of cortisone, as compared to the control. There was a decrease of approximately 100-fold (2 logs) in virus infectivity in the tube containing 10 mg of cortisone per ml. This concentration is greatly in excess of what would be expected within the body of a cortisone-treated mouse. It is not known whether this anti-viral effect was due to cortisone itself or to the suspending and preserving agents in the cortisone suspension (Merck). It is clear that there was no stimulating effect on virus growth resulting from the *in vitro* exposure to cortisone

ACTH. Adrenocorticotropic hormone at a dosage of 0.5 mg every 6 hours for 5 days failed to influence either favorably or unfavorably the course of infection with West Nile or Ilheus virus inoculated intraperitoneally after the 10th dose of the hormone. Similar results have been reported with other viruses(2,3,7,8). However, when the dose was quadrupled, to 4 mg every 12 hours, and administered in

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7. Loosli, C. G., Hull, R. B., Berlin, B. S., and Alexander, E. R., *J. Lab. and Clin. Med.*, 1951, v37, 464.

8. Hull, R. B., and Loosli, C. G., *J. Lab. and Clin. Med.*, 1951, v37, 603.

gelatin to prolong absorption, a cortisone-like effect on virus susceptibility was observed (Table II). Four treatment controls (no virus inoculation) survived more than 2 weeks after this ACTH treatment without apparent ill effect.

Adrenalectomy. The LD_{50} titer for West Nile virus in adrenalectomized mice (approximately 30% were incompletely adrenalectomized) was $10^{-1.6}$ as compared to $10^{-0.6}$ in parallel normal controls. This difference is insignificant, indicating that adrenalectomy had no effect, either adverse or therapeutic, on this virus infection.

Steroids other than cortisone. Testosterone, estradiol, progesterone, and desoxycorticosterone in maximum tolerated dosage appear to have no significant effect on the course of the virus infections (Table III). As judged by the LD_{50} titers, desoxycorticosterone had an apparent slight therapeutic effect (indicated in the table as a negative difference in the logs of the LD_{50} titers) on Bunyamwera virus, but not on West Nile virus. Conversely, progesterone appeared to have an adverse effect on mice infected with West Nile virus, but a very slight therapeutic effect on mice inoculated with Bunyamwera virus. It is felt that these slight and inconsistent differences are not significant.

Clinical studies. Four patients with advanced inoperable cancers were inoculated with West Nile virus while receiving large doses of cortisone. This study was part of a clinical evaluation of the anti-neoplastic effects of several viruses in man(9). West Nile virus produces no subjective evidence of disease in man. Infection is evidenced only by occurrence of viremia for a few days after virus inoculation. Cortisone dosage was 400 mg every 12 hours intramuscularly in 3 patients and 200 mg every 6 hours by mouth in one patient. The drug was given for 2 to 4 days prior to virus inoculation (2.5 ml of 20% infected mouse brain suspension) and was continued for a total of 10 to 14 days. These doses caused rapid fluid retention but no other clinical evidence of toxicity. By clinical standards this is massive cortisone dosage. It probably closely approaches the maximum that can safely be administered to man. Virus infection was established in only one patient and caused no signs or symptoms. Infection with West Nile virus was established 4 times in 18 similar patients without cortisone treatment. Thus, in this limited clinical trial cortisone treatment did not appear to influence West Nile virus infection in man as judged by susceptibility to infection (occurrence of viremia) or clinical course. Obviously, these criteria are in no way comparable to the criteria used in the mouse experiments.

Discussion. In causing this adverse effect on virus infection, cortisone might act directly or indirectly. By direct action it might so affect the virus as to make it more virulent, or it might so affect the host as to make it more susceptible. Such an effect on the host might result from changes in susceptible cells such as to favor virus invasion or propagation, or from interference with cellular and humoral defense mechanisms. Cortisone might act indirectly through its effect on the pituitary and adrenal cortex, thereby causing changes in other hormones which in turn might be the agents directly responsible for the effects observed. From the present data, it appears unlikely that the cortisone effect is dependent on suppression of pituitary or adrenal function, because the other steroid hormones tested did not affect susceptibility to the viruses, and because adrenalectomy did not significantly alter virus susceptibility. Furthermore, if the cortisone effect were due to reduced output of a protective adrenal substance, as has been hypothesized by Schwartzman(3), one might expect that stimulation of the adrenal by ACTH would increase the output of this substance in proportion to the cortisone output and therefore would have a therapeutic effect or no effect at all on virus infection. Since the opposite was observed when very large doses of ACTH were used, it appears that the endogenous cortisone or cortisone-like steroids produced in response to ACTH were unopposed by any hypothetical protective material. Studies by other workers(2,3,7,8) on the effect of ACTH on virus

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9. Southam, C. M., and Moore, A. E., *Am. J. Trop. Med.*, 1951, in press.

infections have shown either no effect or suggestive indications of a cortisone-like effect. The ACTH dosage used by these workers has never equalled, and has usually been much smaller than that used in the present studies. The necessity for large doses of porcine ACTH for adrenal stimulation in the mouse has been emphasized by Kass *et al.*(10). Kalter and coworkers(11) have reported that small doses of ACTH or cortisone decrease, and small doses of testosterone increase, the rate of influenza virus propagation in the mouse for 48 hours after inoculation, but apparently not thereafter. This phenomenon is probably unrelated to that studied in the present experiments. The fact that virus virulence was unaffected by *in vitro* incubation with cortisone, makes improbable the hypothesis that cortisone acts directly on the virus rather than through the host. In summary then, the evidence at hand suggests that the adverse effect of cortisone upon virus infections is a direct result of the action of cortisone itself upon the host. The observation that cortisone inhibits the early stages of the inflammatory reaction(12) suggests a possible mechanism for this effect. It is not yet known if other compounds of the cortisone type (11-oxy steroids) have this same effect on virus infections.

It is of interest that while cortisone treatment rendered mice more susceptible to some viruses, it did not make mice susceptible to Anopheles B virus by the intraperitoneal route. Kilbourne and Horsfall(4) have demonstrated that a Coxsackie virus will infect adult cortisone-treated mice whereas it will not infect normal adult mice. These observations are readily reconcilable if one reasons that cortisone causes merely a quantitative diminution in host resistance. Thus, it may be that cortisone has a marked effect on West Nile, Ilheus, Bunyamwera, and Coxsackie virus infection because with these viruses there is a delicate adjustment between virus invasiveness and host resistance, while Anopheles B virus has insufficient invasive ability to cause death even with the "help" of cortisone. If this hypothesis is correct, one might expect that if a highly virulent virus were studied, cortisone might have little effect on the course of infection because such a virus would be so efficiently invasive that it can readily overwhelm host resistance even without the "help" of cortisone.

Summary. Large doses of cortisone greatly increased the susceptibility of mice to lethal infection by West Nile, Ilheus, and Bunyamwera viruses. ACTH in massive dosage produced the same effect with West Nile virus. Desoxycorticosterone, testosterone, progesterone, and estradiol in massive doses had no significant effect on these virus infections in mice. Anopheles B virus was not infective by intraperitoneal route even in cortisone treated mice. Adrenalectomy did not influence susceptibility to virus infection. The possible mechanisms of the cortisone effect are discussed and it is concluded that the effect of cortisone upon virus infection is probably due to a direct effect of cortisone upon the host.

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Spirocheticidal Activity of Plasma and Serum Fractions.* (18990)

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The treponemal immobilization test by Nelson and Mayer(1) provides a convenient *in vitro* measure of at least one type of circulating antibody against pathogenic *T. pallidum.* The substances measured in the serums of syphilitic patients and animals are apparently the same as the spirocheticidal substances reported by earlier investigators(1,2). While the relationship of these antibodies to acquired immunity in syphilis is by no means clear(3), it is hoped that an understanding of the nature of these spirocheticidal substances will aid in the interpretations of the host reaction to infection. It is established that these "immobilizing antibodies" are associated with the protein fraction of serum; "that they are not reagin;" and that they are relatively heat stable. The present report is concerned with fractionation of syphilitic human serums and plasmas using Cohn's Method 10(4). It will be shown that most of the immobilizing activity is associated with Cohn's fractions III-1,2 which represents the high molecular weight isoagglutinins and a small amount of prothrombin protein, and fibrinogen when plasma was employed, and fraction I + III-3 which represents the high molecular weight antihemophilic globulin and cold insoluble globulin and small amount of plasminogen.

Experimental. Since Method 10 of Cohn was developed for the fractionation of normal human plasmas, minor adjustment in the first step of the procedure was required in order to make it adaptable to syphilitic human serums and plasmas. The aim of such adjustment was to maintain the pH and the ionic strength at the levels specified by the Cohn Method. It was necessary to commence fractionation with an adjusted "Reagent A." The original Reagent A contains 250 ml of 95% ethanol and 2.5 ml of 0.8 $\Gamma/2$ pH 4.0 sodium acetate buffer per liter. Four parts of this reagent when added to one part of Cohn's A.C.D. anticoagulant containing plasma(4) results in a mixture having the desired pH of 5.8-5.9. It was found that for most syphilitic specimens a less acid reagent was required. Thus a series of modified reagents with a lower acetic acid content was prepared and kept in stock. From these reagents a suitable one (furnishing the desired pH of 5.8-5.9) was selected for each syphilitic plasma and serum by "titration." In this procedure 4 ml of the modified Reagent A, cooled to −5°C, was added to 1 ml of plasma in a tube cooled to 0°C. It was necessary to add the reagent in small portions while the tube was rotated and kept at −5°C. 1 ml of the well mixed suspension was diluted with 4 ml of 0.02 M sodium chloride and the pH determined with the aid of a Cambridge pH meter. The other details concerning fractionation were exactly as those given by Cohn(4). The immobilization tests were performed in the manner of Magnuson and Thompson(2). Quantitative tests were controlled with a single pool of positive serum, and titers are expressed as that dilution of serum which immobilized 50% of the organisms after 16 hours incubation at 35°C.

In Table I are recorded the spirocheticidal activities of a series of plasma fractions I + III-1,2 and I + III-3 from patients of early latent syphilis. The results show that in most instances the recovered total activity was over 50%. In one instance (patient No. 239), however, recovery was very low, 15.3%,

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TABLE I. Activity of Plasma Fractions III–1,2 and I+III–3.

Patient No.	Plasma	Fraction III–1,2	Fraction I+III–3	Total activity recovered in %	Positive control
24,881	1:460	1:54	1:100	33.6	1:420
239	1:580	1:31	1:58	15.3	1:495
242	1:80	1:56	1:82	172.5	1:660
247A	1:76	1:16.5	1:23.5	52.5	1:280
247B	1:300	1:84	1:83	55.6	1:500
247C	1:510	1:86	1:220	60.1	1:500
251A	1:235	1:58.5	1:100	68.3	1:210
251B	1:990	1:125	1:420	55.1	1:210

Titers converted to dilutions which immobilized 50% of the spirochetes.

TABLE II. Comparison of Activities of Serums and Plasmas.

Patient No.	Serum	Plasma	Positive control
241	1:45	1:82	1:380
242	1:183	1:80	1:660
244	1:345	1:335	1:280
247A	1:76	1:80	1:280
247B	1:330	1:290	1:500
25601A	1:1100	1:500	1:320
25597B	1:16	1:14.5	1:258
25599C	1:320	1:540	1:320
25600D	1:123	1:66.5	1:320
23815E	1:740	1:250	1:320
251B	1:1280	1:910	1:210

Titers converted to dilutions which immobilize 50% of the spirochetes.

in another instance (patient No. 242) it was too high, 172.5%.

In Table II are compared the spirocheticidal activities of a series of serums and plasmas of patients having early latent syphilis. In order to maintain the same ionic strength and pH, the same quantity of anticoagulant was added to serums as was contained in the plasmas. It may be seen in Table II that there is no appreciable difference between the serum and plasma derived from a given patient. It is evident that clotting elements do not remove any of the spirocheticidal activity.

Since it was found that serum contains as much of the spirocheticidal activity as plasma, in the following experiments fractionation was applied to a series of serums (Table III). The total activities of serum fractions I + III-1,2 and I + III-3 range from 26.2% to 72.3% with an average of 51.6%. The results shown in Table III, being more consistent than those of Table I, would indicate that serum is a more suitable starting material for the concentration and isolation of the spirocheticidal substances than plasma.

In order to maintain the same ionic strength and pH in both, the same volume of anticoagulant was added to the serums as there was present in the plasmas.

Summary. There is no appreciable difference between the spirocheticidal activity of the serum and the plasma from patients with early syphilis. Serums and plasmas were fractionated into eight precipitates by the application of Cohn's Method 10. In the majority of the experiments, more than 50% of the spirocheticidal substances were present in fraction III-1,2 associated with high molecular weight isoagglutinins and small amount of prothrombin protein (and fibrinogen when

TABLE III. Activity of Serum Fractions III–1,2 and I+III–3.

Patient No.	Serum	Fraction III–1,2	Fraction I+III–3	Total activity recovered in %	Positive control
235	1:118	1:56	1:14.5	59.8	1:270
237	1:154	1:102	1:11	72.3	1:270
239	1:502	1:56	1:75	26.2	1:540
241	1:50	1:18	1:11	58	1:380
242	1:185	1:57	1:20	41.6	1:660

Titers converted to dilutions which immobilized 50% of the spirochetes.

plasma was employed) and fraction I + III-3 associated with the high molecular weight antihemophilic globulin and cold insoluble globulin and a small amount of plasminogen.

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Effect of Cortisone and of Aureomycin on Baby Pigs Fed a Vitamin B_{12}-Deficient Diet. (18991)

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Using baby pigs as experimental animals, a vit. B_{12} deficiency has been produced on a "synthetic milk" diet by Neumann *et al.*(1). According to Johnson and Neumann(2), administration of crystalline vit. B_{12} to this diet will completely alleviate the deficiency symptoms. Stokstad and Jukes(3) reported in some experiments a "sparing effect" of aureomycin upon the vit. B_{12} requirement of chicks. Lichtman, Ginsberg, and Watson(4) administered aureomycin to pernicious anemia patients and reported a hematological improvement. Cravioto-Munoz *et al.*(5) state that aureomycin can replace vit. B_{12} in the purified diet of the rat. No work has appeared on the relation of cortisone to nutritional vit. B_{12} deficiency.

The present study was conducted to determine the effect of aureomycin and of cortisone when administered to baby pigs fed a vit. B_{12}-deficient ration.

Experimental. Six Chester White pigs (Exp. I) and 5 Poland China pigs (Exp. II) were removed from their dams at 48 hours of age and individually fed *ad libitum*. The alpha-protein "synthetic milk" ration fed was essentially the same as reported in the vit. B_{12} studies of Neumann *et al.*(6). In addition, 2% of sulfathalidine was added per liter of milk. The method of feeding and care of the animals was similar to that reported previously by Johnson *et al.*(7). In Exp. I two groups were compared. Group I received the basal ration plus intramuscular injections of crystalline vit. B_{12} at a level of 0.8 μg/kg body wt/day. Group II received the basal ration plus 100 mg of aureomycin per kg of dry matter consumed. After 49 days on this diet they were given 30 μg of vit. B_{12} by intramuscular injection for 3 successive days and remained on the experiment 14 days after therapy. The 5 pigs in Exp. II received the basal ration for 39 days. At that time they showed signs of vit. B_{12} deficiency, including a very low reticulocyte count. One pig was continued on the basal ration as a control, while the other pigs were divided into 2 groups. Group IV received 20 mg of cortisone daily by intramuscular injection, and Group V received in their diet 100 mg of aureomycin per kg of dry matter consumed.

Blood samples were taken daily during the treatment period, and reticulocyte counts were determined. The thyroids and tongues of the pigs in Groups IV and V, Exp. II, were removed at autopsy for histological examination.

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TABLE I. Performance of Baby Pigs Given Aureomycin or Vitamin B_{12}.

	Group I Basal + B_{12} (7 wk)	Group II Basal + 100 mg aureomycin/kg dry matter consumed	
		Before therapy (7 wk)	On therapy (2 wk)
No. of pigs	4	2	
Avg initial wt, kg	1.33	1.56	
” final ” ”	13.54	6.23	14.45
” daily gain, ”	.250*	.095	.550
Dry matter consumed (kg/kg gain)	1.26*	1.74	1.04

* Statistically significant, $P<.01$, over Group II at 7 wk.

Results and discussion. Table I gives a summary of the total performance of the 2 groups of pigs in Exp. I. The pigs in Group II which received vit. B_{12} injections during the treatment period showed a very marked reticulocytosis (Table III), a 4-fold increase in food consumption, and a significantly improved growth rate. The exceptionally rapid recovery of these pigs after vit. B_{12} therapy indicates that an uncomplicated, severe vit. B_{12} deficiency did exist.

The average daily gains of the 3 groups in Exp. II are summarized in Table II. Twenty mg of cortisone daily had no effect in alleviating the deficiency symptoms of the pigs in Group IV. In fact, cortisone enhanced the deficiency state, the pigs dying after 8-10 days of cortisone injections. Urinary vit. B_{12} excretion averaged 62 mγ per day following 38 days on the deficient diet and immediately prior to cortisone treatment. After 3 days on cortisone, it had increased to 85 mγ per day. This increase in B_{12} excretion, which is presumably a sequel to increased protein catabolism induced by cortisone, is probably a reason for the aggravating effect of cortisone on the B_{12} deficiency. Group V (Exp. II) differed from Group II of Exp. I in that the aureomycin was not added to the basal ration of Group V until a deficiency state existed. The addition of aureomycin at this time caused no reticulocyte response.

The reticulocyte counts for the various pigs of both experiments are given in Table III.

TABLE II. Response of Vitamin B_{12}-Deficient Pigs to Cortisone and Aureomycin.

	Group III	Group IV	Group V
No. of pigs	1	2	2
Avg initial wt, kg	1.65	1.725	1.785
” daily gain, ” (depletion period)	.213	.163	.190
Avg daily gain, kg (treatment period)	no treatment	cortisone given	aureomycin fed
	.083	.037	.141

TABLE III. Reticulocytes per 100 Red Blood Cells.

Days on test	Group II (Exp. 1) aureomycin in diet		Group III (Exp. 2)	Group IV (Exp. 2) basal		Group V (Exp. 2) basal	
	1e	2e	Basal, 1c	3c	5c	2c	4c
14	—	—	5.2	8.1	5.4	6.9	7
28	3.5	.6	3.4	2.4	1.4	1.7	1.5
35	—	—	1.5	.7	1.2	1.2	1.6
39	—	—	—	.5	1.2	1.2	1.6
42	1.9	2.1					
49	1.1	.5					
Days after treatment	Vit B_{12} treatment		No treatment	Cortisone treatment		Aureomycin treatment	
1	2.1	1.6	.2	1.1	2.3	.3	.3
3	6.2	3.9	.1	1.3	3.6	1.3	1.4
5	5	5.7	.6	.5	1	.2	.3
7	6.6	7.4	0	.5	0	.8	0
9	6.6	12.2		—	.1	.5	0
11		8.2		—	—	.1	.1
13	—	5.2	.1	—	—	.2	0

Hemoglobin and red cell counts were also made at the beginning of the treatment period. The red blood cell counts were essentially normal, but the hemoglobin values were low (avg. 10.6 g %).

Examination of the thyroids and tongues of the pigs in Groups IV and V indicated hypertrophy of these organs as reported previously(8). In addition, in certain areas of the thyroids the follicles were abnormally small and contained little colloid.

Summary. A vit. B_{12} deficiency has been produced in baby pigs fed an alpha-protein "synthetic milk" diet. The addition of aureomycin to this diet, either before or after the deficiency state existed, did not give a beneficial response. The injection of 20 mg of cortisone daily into vit. B_{12}-deficient pigs enhanced the deficiency state rather than having any curative effect.

We are indebted to Merck and Co., Rahway, N. J., for crystalline vitamins and cortisone; to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., for folic acid and aureomycin hydrochloride; and to Sharp and Dohme, Glenolden, Pa., for sulfathalidine.

We also wish to acknowledge the help of Margaret B. Keane for carrying out the reticulocyte counts, of Dr. Marian F. James for histological examination of the thyroids and tongues of the deficient pigs, and of Dr. S. W. Terrill of the Swine Production Division for providing the baby pigs used in this work.

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Blood and Plasma Volume and Thiocyanate Space of Normal Chicks.* (18992)

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(Introduced by F. J. Stare.)

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The blood and plasma volumes and thiocyanates space of humans, dogs and rats have been investigated by various authors but no data appear to have been collected on chicks. In connection with studies on high salt diets which will be published subsequently we have determined the volumes of these fluid spaces in normal White Leghorn cockerels of various ages. The ease with which intra-jugular injections may be made in this species may make it a useful species for such studies.

Methods. The methods used have been previously described by Wang and Hegsted (1) in studies with rats. A mixture of Evans Blue Dye and ammonium thiocyanate solution was injected into the jugular vein of the chick under light ether anesthesia. Ten minutes later a blood sample was taken with a heparinized syringe by heart puncture. The hematocrit was determined with the small Van Allen tubes and the thiocyanate and Evans blue concentrations in the blood determined as previously described. From 0.2 to 0.3 ml of solution containing 5 mg of dye and 40 mg of thiocyanate per ml was used depending upon the size of the chick. The concentrations in the solution injected were determined by analysis after the addition of a known amount to a control sample of chick blood. Preliminary studies showed that ten minutes after injection was sufficient time to yield essentially stable blood concentrations. When several chicks were to be studied, it was convenient to inject 4 chicks at approximately 2½ minute intervals and bleed them during

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* Supported in part by grants-in-aid from the Swift & Co., Chicago, Ill. and the Nutrition Foundation, Inc., New York.

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TABLE I. Mean Values Obtained with Chicks of Various Ages.

Age, days	No. of chicks	Body wt, g	Plasma vol, % B. wt	Blood vol, % B. wt	Thiocyanate space, % B. wt	Hematocrit, %
13	7	121	6.7	9.9	40.7	32.2
20	6	190	6.3	9.1	39.9	31
27	7	279	7.2	10.4	43.8	30.6
35	12	411	6.4	9	46.2	30.4
Mean ± Stand. error of mean			6.56 ± .15	9.55 ± .21	43.28 ± .78	30.47 ± .20

the subsequent 10 minutes.

Results. The mean values obtained from 32 chicks which were examined in groups of different ages are shown in Table I. The values are essentially constant when expressed as percentage of body weight regardless of the age or weight of the chick. In earlier studies with rats(2), most of the fluid volumes did not remain constant at different ages when expressed as percent of body weight. However, only a relatively small range of age has been studied with chicks and these values probably will not hold for much older birds. If the present values are compared to those obtained with young rats(2), the blood volume is approximately the same but the plasma volume is larger because of the smaller red cell volume. The thiocyanate space is much larger than was found in rats and is similar to that obtained by Fellers *et al.*(3) for very small babies. This might be expected since the smallest rats studied, 60-to-70 grams, are much older actually and relatively than the chicks used in this work. Data on suckling rats which might be comparable have not been reported.

Summary. The blood and plasma volumes and thiocyanate space in normal chicks from 13 to 35 days of age have been determined.

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Demonstration that Para-aminohippuric Acid "Enters" the Human Erythrocyte.* (18993)

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Para-aminohippuric acid (PAH) has been found to penetrate the canine erythrocyte(1), but its entrance into the human erythrocyte has been denied(2-4). This report demonstrates that PAH does "enter" the erythrocyte in man.

Recent studies in this laboratory of renal blood flow (RBF) in man included a com-

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[*] The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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[¶] Mailing address: c/o American Embassy, Cairo, Egypt.

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parison of the RBF determined by renal extraction (E) of PAH from whole blood with the RBF determined by E from plasma and the hematocrit. The latter calculation was noted to be consistently higher than the former in experiments in which the renal venous plasma was not separated from the cells immediately. In consequence the ratio of PAH in the whole blood cell water to that in the whole blood plasma water was determined. The experiments cited below show conclusively that PAH is present in the cellular fraction of whole blood. No evidence is presented for its actual location but its presence in the erythrocyte is assumed for simplicity of discussion. The practical point is the observation that PAH enters the cellular fraction, not the specific type of cell or cells involved. Since PAH is almost completely extracted from the plasma passing through normal human kidneys(5), extraction will be falsely low and renal plasma flows (RPF) will be falsely high if cell-plasma separation is delayed sufficiently to allow PAH to diffuse from the cells into the plasma. The unlikelihood of PAH extraction from the erythrocyte during the passage of blood through the kidney has been discussed previously(1).

Methods. (1) *Technics.* The analytical technics employed were the same as those previously described(1) except that in the present studies a Beckman DU Spectrophotometer was used. Arterial samples were drawn from an indwelling needle(6) and simultaneous renal venous samples were obtained from an indwelling catheter(5). After the priming infusion of PAH, a sustaining infusion was maintained for at least one hour before collection of the first blood and urine samples. This was done in order to assure a steady state. Observations for several hours at arterial plasma levels of PAH *circa* 2 mg % have been analyzed (Table I). In other experiments (Table II) arterial plasma concentration was elevated to 80-100 mg % for an hour or more before the first blood and urine samples were obtained. After these were taken the sustaining infusion was changed to one containing no PAH, and blood and urine samples were collected over the subsequent 2-3 hours. (2) *Calculations.* Hematocrits were determined as described previously(1) after centrifugation for one hour in Wintrobe tubes. The correction factor of Chapin and Ross(7), 8.5%, was applied to the observed cell column to obtain true cell volume (V_c). The true plasma volume (V_p) per volume of whole blood = $100 - V_c$. From the plasma and whole blood specific gravities(8), values for the hemoglobin concentration in whole blood (Hb_b) and the protein concentration in the plasma ($Prot_p$) were obtained. The hemoglobin concentration in the cells (Hb_c) was obtained from the formula $Hb_c = \frac{Hb_b}{V_c} \times 100$.

The apparent specific volume of protein, both plasma protein and cellular hemoglobin, was assumed to be 0.74(9). Values for cell water (C_{H_2O}) and plasma water (P_{H_2O}) are not included in the tables but were calculated as $100 - (Hb_c \times 0.74)$ and $100 - (Prot_p \times 0.74)$, respectively. The PAH content of the plasma fraction of 1 liter of whole blood was obtained by multiplying the plasma concentration by $\frac{V_p}{100}$. This value subtracted from PAH concentration in whole blood was recorded as the PAH content in the cell fraction of the whole blood. The cell fraction value divided by $\frac{V_c}{100}$ was taken as the cell concentration in mg/l. The PAH concentration in cells $\times \frac{100}{C_{H_2O}}$ was taken as the PAH concentration in cell water ($PAH_{c_{H_2O}}$). $PAH_{p_{H_2O}}$ was obtained by substituting p values for c

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TABLE I. Data Demonstrating That PAH "Enters" the Human Erythrocyte at Low Plasma Arterial PAH Concentrations.

						PAH concentrations in mg/l							
Sample	Time, min	V_c cc/100 cc	V_p cc/100 cc	Hb_c g/100 cc	$Prot_p$ g/100 cc	Whole blood	Plasma	Cells	Plasma fraction of whole blood	Cell fraction of whole blood	Plasma water	Cell water	$\frac{PAH_c H_2O}{PAH_p H_2O}$
A2	130	33.8	66.2	32.9	7.1	17.15	25.21	1.33	16.70	.45	26.62	1.76	.066
3	142	33.3	66.7	32.8	7.2	17.26	25.74	.27	17.17	.09	27.18	.36	.013
	149-159		1 liter of dextran infused										
4	170	28.2	71.8	33.2	7.05	14.55	19.86	1.06	14.25	.30	20.93	1.41	.067
5	180	28.4	71.6	33.6	7.05	16.12	22.09	1.09	15.81	.31	23.29	1.45	.062
6	283	32.4	67.6	34.6	6.6	20.27	29.29	1.45	19.80	.47	30.80	1.95	.063
7	297	32.4	67.6	34.6	6.6	20.42	29.49	1.51	19.93	.49	31.00	2.03	.065
RV2						1.38	1.54	1.06	1.02	.36	1.63	1.40	.859
3						1.38	1.54	1.05	1.03	.35	1.63	1.38	.847
4						.98	1.28	.21	.92	.06	1.35	.28	.207
5						.98	1.28	.21	.92	.06	1.35	.28	.207
6						1.68	1.87	1.26	1.27	.41	1.97	1.69	.858
7						1.80	1.87	1.64	1.27	.53	1.97	2.20	1.116

TABLE II. PAH Concentration in Human Erythrocyte at High Plasma Arterial PAH Concentrations; Demonstration of the Slow *In Vivo* Diffusion of PAH from Cells to Plasma.

						PAH concentrations in mg/l							
Sample	Time, min	V_c cc/100 cc	V_p cc/100 cc	Hb_c g/100 cc	$Prot_p$ g/100 cc	Whole blood	Plasma	Cells	Plasma fraction of whole blood	Cell fraction of whole blood	Plasma water	Cell water	$\frac{PAH_c H_2O}{PAH_p H_2O}$
A 2	183	38.2	61.8	35.9	7.33	588.5	874	127	540	48.5	923	173	.188
13	326	35.7	64.3	35.2	6.37	29.4	22.2	42.45	14.25	15.15	23.3	54.4	2.46
	332-343		1 liter 6% albumin infused										
15	433	30.7	69.3	36.4	6.67	481.5	652	96	452	29.5	686	131	.191
27	566	30	70	36.7	6.48	22.35	18.5	31.35	12.95	9.4	19.45	43	2.21
RV 2						459.5	656	142	405	54.5	693	193.5	.279
13						17.95	4.35	42.45	2.8	15.15	4.57	57.4	12.55
15						368	505	60	349.5	18.5	531	82	.154
27						11.75	2.9	32.35	2.05	9.7	3.05	44.4	14.55

values in this paragraph. (3) *Other points.* Specific gravity and hematocrit determinations were done only on arterial blood and these values were assumed for the renal venous samples. No great error could arise from this practice in these experiments for urine flow was usually less than 0.3% of RBF. The highest ratio obtained when the urine flow was 9 cc per minute and RBF was over 1800 cc per minute. Validation of the accuracy of the plasma and whole blood analyses was proven by a technic to be described elsewhere(10). The other essential point was the manner of handling the renal venous samples. They were quickly drawn (15-30 seconds) into chilled, oiled syringes, transferred rapidly to lusteroid tubes containing a dry film of heparin, inverted a few times, centrifuged in a refrigerated centrifuge at 2800 rpm for 5 minutes and the plasma immediately removed from the cells.

Results and discussion. In arterial blood at low PAH concentration, *circa* 3 mg per 100 cc, the ratio $PAH_{c_{H_2O}} : PAH_{p_{H_2O}}$ averaged about 0.06 in over 20 bloods from 4 patients; the ratio in simultaneously drawn renal venous samples was over 0.8. Representative examples are given in Table I. The inherent error (up to 1%) in the analytical methods would not permit detection of lower ratios.

Proof that PAH does "enter" the human erythrocyte does not depend solely on the results obtained from calculation of the ratio $PAH_{c_{H_2O}} : PAH_{p_{H_2O}}$. In Table II it will be noted in both arterial and renal venous samples numbered 13 and 27 that the whole blood actually contained more PAH than did the plasma. In this instance the values are well beyond experimental error, and similar results have been observed in other experiments.

Once PAH has entered the erythrocyte it leaves it slowly, *cf.* arterial values in the last column of Table II. It is obvious from comparison of the final column of Tables I and II that in the experiment cited in Table II, the diffusion gradient from cells to plasma in renal venous blood when the arterial plasma PAH has reached 2 mg % has been increased 10-20 fold over that prevailing in the steady state, Table I, and that the cell concentration of PAH in renal venous blood is 200 times that which will be present when diffusion equilibrium is attained.

An example of the magnitude of error that will occur in plasma extraction values if renal venous plasma is not separated immediately is taken from the experimental data in Table II. If renal venous samples 13 and 27 were allowed to stand until diffusion of PAH from cells to plasma has proceeded to the point where the ratio in the simultaneously drawn arterial sample prevailed, then the observed PAH plasma extractions would be 23 and 24% respectively, or 28.5% of the observed values in this individual with mild renal damage. Likewise calculation of RPF would be nearly 4 times the true value.

Conclusions. (1) Contrary to previous reports in the literature para-aminohippuric acid (PAH) has been found to "enter" the human erythrocyte at low and high plasma PAH concentrations. (2) It is pointed out that PAH plasma extraction will be falsely low and renal plasma flow estimation from the extraction values will be falsely high unless renal venous plasma is separated *as soon as is possible* after the blood leaves the kidneys. An alternate procedure is to determine the whole blood extraction and estimate the plasma extraction with the aid of the hematocrit.

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Intravenous Treatment of Pernicious Anemia with Citrovorum Factor (Leucovorum).*† (18994)

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There is ample evidence that the intramuscular administration of citrovorum factor (CF) will induce hematologic and clinical remissions in patients with pernicious anemia, nutritional macrocytic anemia and sprue(1-4). The present study was undertaken to determine whether the intravenous injection of this vitamin would induce similar results. Two patients with typical Addisonian pernicious anemia in relapse were treated with 3.0 mg of CF intravenously, daily, for 41 and 78 days, respectively. Each case showed hyperchromic macrocytic anemia, histamine-fast achlorhydria, and megaloblastic bone marrow. Roentgen examinations of the gastro-intestinal tract, urinalyses and blood chemistry determinations disclosed no abnormalities. Hemoglobin and red blood cell determinations were made 3 times a week, leukocyte counts once a week, and reticulocyte estimations daily.

G. F., white male, age 63, was admitted to the hospital with the chief complaints of progressive weakness, anorexia, pallor, parasthesias of arms and legs, dyspnea and precordial pain on exertion of three months' duration. The positive findings on physical examination were: evidence of recent weight loss, yellowish pallor of skin and mucous membranes, smooth tongue, apical systolic murmur, spleen palpable one cm below the left costal border and enlargement of prostate gland. Neurologic status was entirely normal. The initial hemoglobin and red blood cell values were 8.0 g per 100 ml and 2.14 million per cmm, respectively. He was given 3.0 mg of CF intravenously, daily, for 41 days. Improvement in sense of well being, appetite and strength were observed on the seventh day and continued upward during the course of treatment. A submaximal reticulocyte peak of 9.6% was noted on the ninth day of treatment. At the end of this period of observation it was found that the hemoglobin and erythrocytes had reached stationary levels of 12.0 g per 100 ml and 3.86 million per cmm, respectively. Accordingly, the route of administration was changed to the intramuscular one. This resulted in a rise of blood values to normal levels in 6 weeks (Fig. 1). The patient appeared clinically well and returned to work. No nervous system changes were observed.

I. R., white female, age 74, was a known case of pernicious anemia of four years' duration who was successfully treated with the parenteral administration of liver extract. For the past year the patient failed to attend the clinic and received no specific therapy. At the time of the present admission her symptoms were mild and were mainly those of weakness and fatiguability. Physical examination was entirely negative. Neurologic survey revealed hyperactive deep tendon reflexes and positive Babinski reaction on the right side. The initial hemoglobin and red blood cell values were 11.5 g per 100 ml and 2.84 million per cmm, respectively. She received 3.0 mg of CF intravenously, daily, for 78 days. Clinical improvement started on the eighth day with an increase in appetite and sense of well being and continued during the entire period of observation. No reticulo-

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† Supplied by Dr. J. M. Ruegsegger, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

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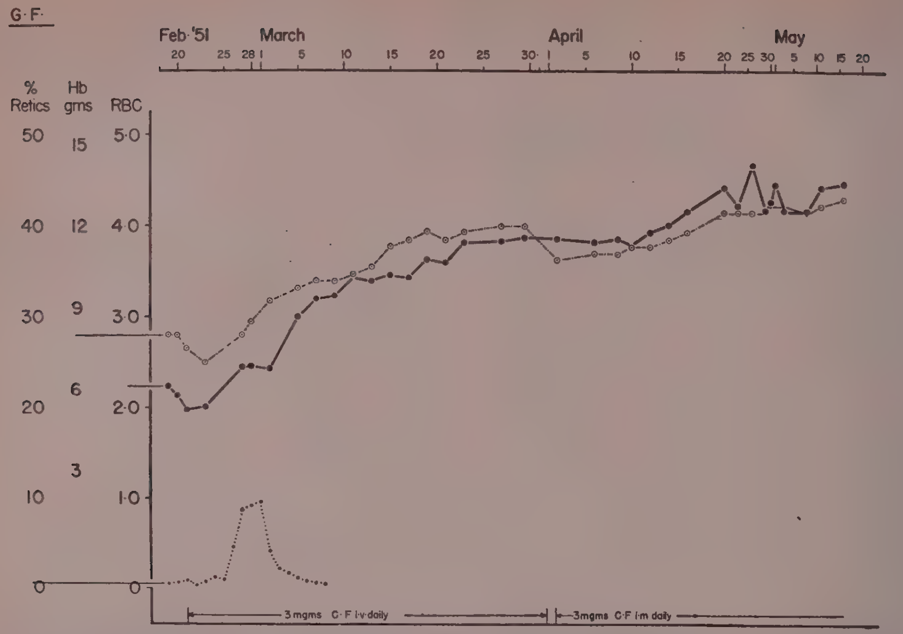

FIG. 1. Hematologic data on patient G. F. with pernicious anemia treated with daily intrav. inj. of 3 mg of CF. Reticulocyte response was submaximal and blood levels were suboptimal. Administration of same dose intramuscularly induced a normal blood count.

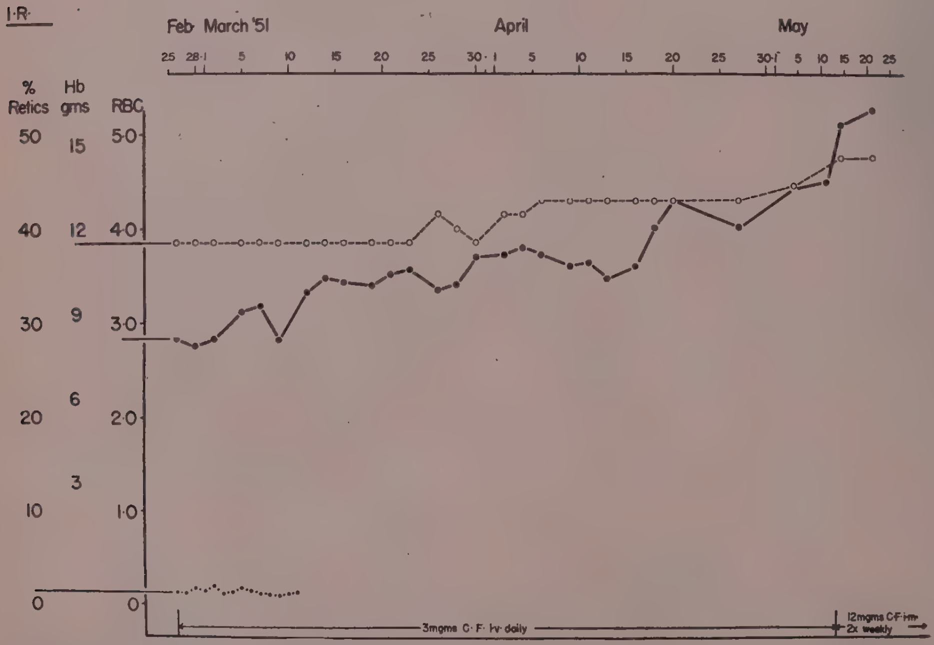

FIG. 2. Hematologic data on patient I. R. with pernicious anemia treated with daily intrav. inj. of 3 mg of CF. No reticulocyte response was observed. The hemoglobin and erythrocytes rose to normal levels in 68 days.

cyte response was noted. The blood values reached normal levels on the 68th day. Ten days later the patient was placed on 12 mg of CF intramuscularly, twice weekly. At the end of 117 days she appeared entirely well (Fig. 2). No alteration in neurologic status was noted.

Discussion. From the foregoing data it is clear that intravenously administered CF in doses of 3.0 mg a day induced clinical remissions and a moderately good to a complete hematologic response in two patients with pernicious anemia in relapse. It would appear that in one case (I. R.) the excretion rate surpassed the utilization rate because when the route of administration was changed to the intramuscular one the hemoglobin and erythrocytes rose to normal levels. There was no instance of development or progression of nervous system changes in either patient after 3 to 4 months although one of them (I. R.) showed evidence of spinal cord disease before treatment was begun. Recently we have observed rapidly developing alterations in the central nervous system after 3 months of oral treatment of a patient with pernicious anemia using 6.0 mg of CF daily(5). The present hematologic results also differ from our earlier report in that in this group normal blood counts were attained(2). The reticulocyte response was absent or submaximal in the 2 cases presented and this confirmed the observations made when patients were treated by the intramuscular route(2).

Summary. The daily intravenous administration of 3.0 mg of CF to two patients with pernicious anemia in relapse was followed by a satisfactory clinical remission, poor reticulocyte response, suboptimal hemoglobin and red blood cell levels in one case and a normal blood count in the other. The subsequent intramuscular injection of the same dose to the first patient resulted in a good hematologic remission.

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Effect of Intravenous Aureomycin on the Intestinal Flora of Dog and Man.* (18995)

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(Introduced by Jacob Fine.)
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With increasing experience it is becoming evident that the toxic effects of aureomycin on the gastrointestinal tract may occur when the drug is given intravenously as well as orally(1,2). Since this may be due to excretion of the drug into the gastrointestinal tract, its administration parenterally may exert an antibacterial effect on intestinal flora. This report deals with a study of effects of intravenous aureomycin on the normal intestinal flora of dog and man.

Effect of aureomycin on intestinal flora of dogs. Method. Sixteen healthy mongrel dogs† received aureomycin intravenously daily for a period of 10 days. Four of them were given 1000 mg, six 600 mg, and six 400 mg, in 2 equal doses each day. The bacterial flora of the stools in all dogs was examined(3) before treatment was started, daily during the course of treatment, and for a few days thereafter.

Results. (a) 1000 mg/day. This dose proved to be toxic, as previously reported(4). Two of 4 dogs died in 3 days without any

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* Aided by a grant from Lederle Laboratories Division, American Cyanamid Co.

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2. Rutenburg, A. M., unpublished data.

† Each weighed about 10 kg.

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demonstrable change in the fecal flora. The other 2 died in 5 days. One of these showed no coliform bacilli in the stool; the other showed a reduction to about 1/150 of the original count.

(b) 600 mg/day. All 6 dogs in this group survived, although by the 7-8th day they stopped eating and lost weight. Recovery was rapid following cessation of therapy. In 4 dogs the coliform bacilli disappeared from cultures in 7-8 days, and in the remaining 2 the coliform count was markedly reduced. Strains of *E. coli* still present after 10 days of treatment were sensitive *in vitro* to aureomycin.

(c) 400 mg/day. The number of coliform organisms was definitely reduced in all 6 dogs, but in only 2 of the 6 were the bacilli completely eliminated after 10 days of therapy.

In all 16 dogs the Clostridia and Enterococci were moderately reduced, but did not disappear from cultures.

Comment. These results and data reported elsewhere(3) indicate that intravenous aureomycin has a definite antibacterial action on the normal fecal flora of the dog, but its suppressive effect was inferior to that of the oral preparation.

Effect of aureomycin on intestinal flora of man. Method. Twenty-five patients with a variety of surgical infections received 1000 mg of aureomycin intravenously in 2 equal doses for 3 to 25 days. No other antibacterial agent was given to these patients. The stools were cultured before, during, and occasionally, after cessation of therapy.

Results. In 15 of the 25 patients the coliform bacilli and Enterococci disappeared from the stools in about 3 to 7 days. The Clostridia were completely suppressed in only 7 of the 15, and markedly reduced in 2 others. In 5 other patients there was a marked reduction in the counts of the coliform bacilli, Clostridia, and Enterococci. The remaining 5 showed no change in the coliform counts but partial to complete suppression of Clostridia in all, and partial suppression of Enterococci in 2. There was no reduction in the number of colonies of *B. proteus vulgaris* or *Pseudomonas* in any of the 25 patients.

Toxicity. Gastrointestinal side effects occurred in 8 patients (31%). Of 6 patients with diarrhea, 2 had severe protracted diarrhea which upset the fluid and electrolyte balance. Two other patients had nausea and vomiting. No other toxic effects were observed.

Comment. The results indicate that intravenous aureomycin is more effective in man than in the dog in the suppression of the normal intestinal flora. Thus, coliform organisms were completely or partially suppressed in 20 patients: Clostridia in 19; and Enterococci in 22 of the 25 patients.

Discussion. The suppression of the fecal flora and the occurrence of gastrointestinal side effects following the administration of aureomycin intravenously is presumably due to the excretion of the drug into the gastrointestinal tract in man via the bile and salivary glands. Aureomycin administered intravenously has been shown to be excreted in bile in high concentration(5,6); and the oral lesions following intravenous aureomycin suggest excretion in the saliva(2).

The inferior antibacterial action of aureomycin on the intestinal flora of the dog as compared to man may be related to the much higher concentration of sodium taurocholate in the bile of the dog(7). It is notable that following oral therapy, the effect of aureomycin on the intestinal flora is identical in both man and dog(3,8).

Summary. 1. Aureomycin administered intravenously results in partial suppression of the intestinal flora in both man and dog. The effect in patients is superior to that in dogs. 2. The drug is presumably excreted into the intestinal tract via the salivary glands and bile.

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Catabolism of I^{131} Labelled Bovine Gamma Globulin in Immune and Non-immune Rabbits.* (18996)

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In contrast to antigens such as pneumococcal polysaccharides(1,2), azo dye labelled proteins(3-6), rickettsia and viruses(7), which remain intact in the host's tissues for long periods of time, heterologous serum protein antigens appear to persist in the host's tissues for only a relatively short time(8,9). These heterologous serum proteins are not eliminated as such in appreciable amounts by the host but rather are catabolized. It has already been noted that this antigen catabolism is more rapid in immune animals than in non-immune (9).

The present study was undertaken to learn more about the catabolism of heterologous serum protein antigens with particular reference to the role played by specific antibody. We measured the rate of catabolism of I^{131} labelled bovine gamma globulin† (I*BGG) in non-immunized, actively immunized and passively immunized rabbits. Catabolic rates in non-immunized and actively immunized animals were compared in order to amplify the previously reported observation of increased rate of antigen breakdown in immunized animals. Catabolic rates in actively and passively immunized animals were compared in an attempt to learn whether factors other than the presence of specific antibody were necessary for the increased rate of immune antigen catabolism.

The I^{131} antigen label used in these experiments is well suited to antigen catabolism studies. As previously described, I^{131} can be attached to protein antigens without detectably altering antigenic specificity(9). *In vivo* the I^{131} antigen appears to retain its label as long as it is immunologically active. As the I^{131} protein antigen is catabolized, there is liberation of non-protein bound I^{131}, largely iodide, with small amounts of organic bound forms, both of which are rapidly excreted in the urine(9). Therefore, following the injection of I^{131} labelled antigens, the non-protein bound I^{131} in the body at any time plus the non-protein bound I^{131} excreted up to that time would represent the amount of I^{131} antigen catabolized.

Materials and methods. Three groups of young male albino rabbits weighing from 1.9 to 2.2 kg were prepared as follows: Six rabbits were actively immunized by 2 intravenous injections of 100 mg of BGG 13 and 6 days before the experiment. Five rabbits were passively immunized by 3 injections of rabbit anti I*BGG antibody: 62 mg intramuscularly 24 hours before antigen injection, followed by 15 mg intravenously and 15 mg intramuscularly one hour before. The total amount of antibody passively administered to each rabbit was calculated to be approximately equal to the amount of antibody in the actively immunized rabbits. Six untreated rabbits were used as controls. All rabbits were pre-fed nonradioactive iodide to prevent uptake of liberated I^{131} by the thyroid. Antibody used in passive immunization was prepared by three injections of 75 mg BGG into donor rabbits, which were then bled out 9 days after

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* This investigation was supported by the Atomic Energy Commission, Contract Nos. A(11-1)-55 and AT(30-1)-1205.

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† The bovine gamma globulin was prepared by Armour Laboratories, Chicago, Ill.

the last antigen injection. The concentrated antibody solution used for injection contained 3.1 mg anti I*BGG/cc, as determined by quantitative precipitation. The preparation of the test antigen, I*BGG, and the methods for protein bound and non-protein bound I^{131} determinations have been previously described(10).

Experimental procedure. The rabbits of all 3 groups were given intravenously 37 mg of I*BGG containing approximately 500 μc I^{131}. Protein bound and non-protein bound I^{131} determinations of blood and urine were made 1, 6, 24 and 48 hours after injection of antigen. The rabbits were kept in metabolism cages for urine collection, and their bladders were emptied by catheter at each stated time interval to insure accurate urine collection. The total body non-protein bound I^{131} was estimated by multiplying the amount found in one cc of whole blood by 37% of the body weight expressed in grams. This factor for estimating total body non-protein bound I^{131} was obtained by measuring the dilution of I^{131} in the blood stream following the intravenous injection of blood containing I*BGG breakdown residues into animals with both ureters ligated to prevent excretion of I^{131}.

Results. Both the urinary non-protein I^{131} and the total (urinary and body) non-protein I^{131} are represented for each group of rabbits in Fig. 1 to 3. The accumulative non-protein bound I^{131} values are plotted as percentages of original I*BGG injection against time in hours.

In Fig. 1 (non-immunized rabbits) the I*BGG catabolism with liberation of non-protein bound I^{131} is shown to be relatively slow: a total of 21% had been catabolized at 24 hours and 34% at 48 hours. The amount of non-protein I^{131} found in the urine was slightly less than one-half the total catabolized at the 6, 24, and 48-hour determinations.

Fig. 2 (actively immunized rabbits) shows a rapid liberation of non-protein I^{131} as seen at the 1- and 6-hour determinations. At 6 hours nearly 60% of the injected I*BGG had apparently been catabolized; at 24 hours, 70%; and at 48 hours, 82%. The renal elimination of non-protein I^{131} lagged for the first 24 hours, following which rapid excretion took place.

In Fig. 3 (passively immunized rabbits) the total and urinary non-protein I^{131} curves are practically identical to those for the actively immunized group. Both the rapid I^{131} liberation during the first 6 hours and the 24-hour delay in urinary excretion are apparent.

Discussion. It is evident that the catabolism of I*BGG in immunized animals is much more rapid than in non-immunized animals, especially within the first 6 hours following antigen injection. During this period approximately 60% is catabolized in immune animals and only 7% in non-immune.

Because the rate of I*BGG catabolism is almost identical in both actively and passively immunized rabbits, it appears that nothing

FIG. 1, 2, 3.

Liberated non-protein bound I^{131} plotted as % of I^{131} in original I^{131} BGG injection against time in hr. Dotted lines indicate non-protein I^{131} in urine and solid lines indicate total non-protein I^{131} in both urine and body. Light lines represent individual animals and heavy lines the group averages. FIG. 1—non-sensitized rabbits. Fig. 2—actively sensitized rabbits. Fig. 3—passively sensitized rabbits.

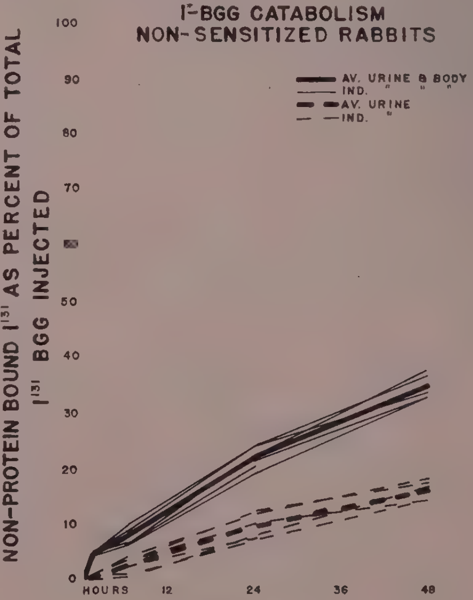

FIG. 1.

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10. Talmage, D. W., Dixon, F. J., Bukantz, S. C., and Dammin, G. J., in press.

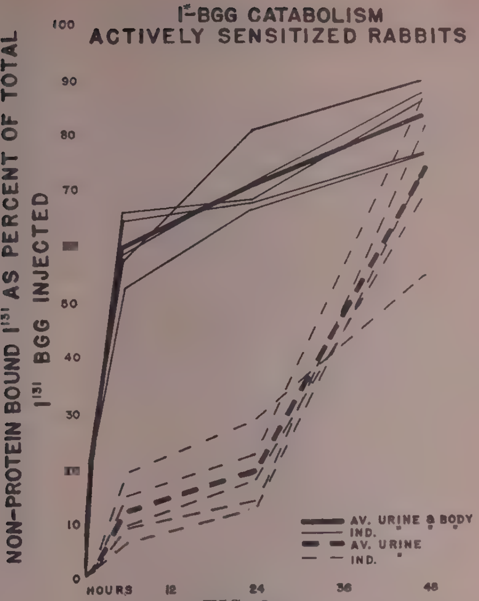

FIG. 2.

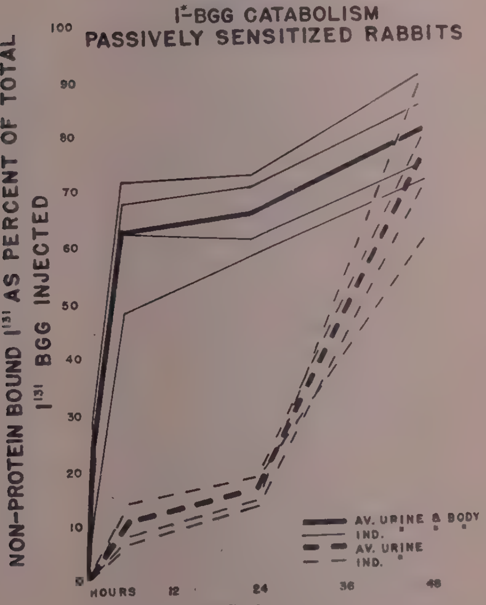

FIG. 3.

other than the presence of specific antibody is necessary for rapid immune antigen catabolism. Whether or not any cellular enzymatic adaptation which facilitates antigen catabolism occurs during the process of active immunization cannot yet be stated definitely. However, there is no evidence in this experiment of such a change.

The fact that only antibody is necessary to change slow non-immune antigen catabolism to rapid immune catabolism offers several clues to the mechanism of immune antigen catabolism. It seems most likely that the presence of antibody would result in antibody-antigen complexes which would be taken up by phagocytic cells. The phagocytic cells would in turn rapidly catabolize the antigen (and perhaps the entire complex) with liberation of non-protein I^{131}. This mechanism fits in with the observed slow non-immune antigen catabolism where, in the absence of specific antibody, no antigen complexes large enough to stimulate phagocytosis form.

As for the slow non-immune catabolism, it is most likely that the I*BGG enters the plasma protein pool, has free access to all tissues, and is utilized by the tissues, as is the native serum protein(11). It is of interest that the half-life of I*BGG in the non-immune rabbit as measured by liberation of non-protein I^{131} is about 3 days, which agrees well with the half-life arrived at by measurement of antigen disappearance from the blood(10).

Conclusions. 1. The catabolism of I*BGG in immune rabbits is much more rapid than in non-immune rabbits, especially within the first 6 hours. 2. Only specific antibody is necessary for the rapid immune catabolism, as indicated by the identical rates of I*BGG catabolism in actively and passively immunized rabbits.

The authors gratefully acknowledge the competent technical assistance of Misses Maria Deichmiller and Gloria Tomsen.

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Placental Transmission of Mumps and Streptococcus MG Antibodies. (18997)

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(Introduced by G. Shwartzman.)

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Mumps may occur at any age, but it is extremely rare in the neonatal period. This has been said to be probably due to the placental transmission of mumps antibodies(1). As a result of the recent development of specific serological tests(2,3) it has now become possible to obtain quantitative data concerning the permeability of the placenta to these substances.

The present communication embodies the results of testing maternal and cord blood for mumps-complement-fixing and agglutination-inhibiting antibody content. Twenty-five sets of sera were available for the former test, and 47 sets for the latter. A small proportion of the sera were from mothers who delivered prematurely. The results with these were essentially similar to those found with sera obtained from mothers who delivered at term. The results which follow represent consistent findings in 2 or more tests with each specimen.

Methods. The technic for the complement fixation was essentially that of the Kolmer test and has been described elsewhere(4). The antigen was a viral antigen prepared in Lederle Laboratories from allantoic fluid infected with the Habel strain of mumps. The agglutination-inhibition technic previously described(4) is based on the fact that mumps virus agglutinates chicken cells and that mumps convalescent serum in high dilution inhibits specifically this agglutination. For these tests the antigen was prepared in our own laboratory from allantoic fluid infected with the Enders strain of mumps virus. The streptococcus MG agglutination test consisted of making serial dilutions of serum in saline and adding to each a standard suspension of heat-killed organisms. The agglutination readings were made after 24 hours at room temperature(5). In each instance both maternal and cord bloods were tested at the same time.

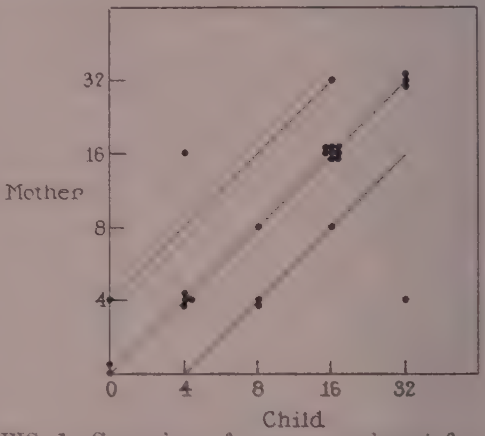

FIG. 1. Comparison of mumps complement fixation titers in maternal and cord bloods.

Results. With the complement fixation test 23 of the 25 mothers (approximately 90%) had antibody titers of 1:4 or above. In 18 sets both sera had exactly the same titer, and in 5 sets there was a difference of only a single dilution (Fig. 1). Thus, within the limits of technical error, in 23 of 25 instances at the time of delivery, the antibody content in the placental blood was quantitatively that of the mother.

With the agglutination-inhibition test, the results were similar but more marked. Forty-seven sets of sera were tested. In 41 both specimens had identical titers. The remaining

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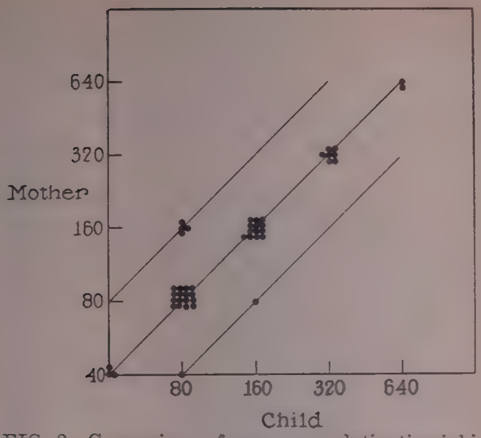

FIG. 2. Comparison of mumps agglutination-inhibition titers in maternal and cord bloods.

6 varied only by a single dilution (Fig. 2). Therefore, with the agglutination-inhibition test, again within the limits of technical error, there was complete agreement between maternal and placental antibody levels.

The transmission of these 2 mumps viral antibodies from mother to child without loss is similar to what is known to be true for diphtheria and tetanus antitoxins(1). These results confirm an earlier report by Maris, Enders, Stokes, and Kane(6) concerning the transmission of mumps complement fixing antibody to 5 infants.

These observations are in contrast to what was found with 25 sets of sera when they were tested for the presence of Streptococcus MG agglutinins. Streptococcus MG is an indifferent streptococcus, first described by Thomas, Mirick, Curnen, Ziegler, and Horsfall(5) which is serologically related to the *Streptococcus salivarius*. It was originally recovered from the lungs of patients dying of primary atypical pneumonia. Agglutinins for it have been found in relatively high titer in the blood of slightly less than one-half of patients convalescing from primary atypical pneumonia(7). It has been found in low titer in the sera of a number of apparently normal individuals. It was therefore selected for comparison with mumps antibodies since it probably also represents a naturally acquired antibody, though to a bacterium rather than to a virus.

There were 20 mothers who had detectable Streptococcus MG agglutinins. In 16 of these 20 sets of sera (approximately 80%) there was a 4-fold or greater *decrease* in titer between maternal and cord blood (Fig. 3). Unfortunately in no instance was the maternal titer greater than 1:16. Consequently it was not possible to show more dramatic differences. This relatively low level of agglutinin may leave some question as to its significance. Nevertheless, it is clear that unlike the mumps antibodies it is not readily transmitted across the placenta.

Our results with the streptococcus MG are not unlike what was found by Hodes, Ziegler, and Zepp(8) when they studied the placental transmission of pneumococcus Type I antibodies. They examined sera from convalescent mothers and from mothers who had been vaccinated with the pneumococcus polysaccharide. In all the mothers the antibody content at the time of delivery was considerably greater than in the infant cord blood.

Discussion. The high incidence of mumps complement fixing antibody in maternal blood, even though only 11 of the 25 mothers tested definitely recalled having had the disease, is

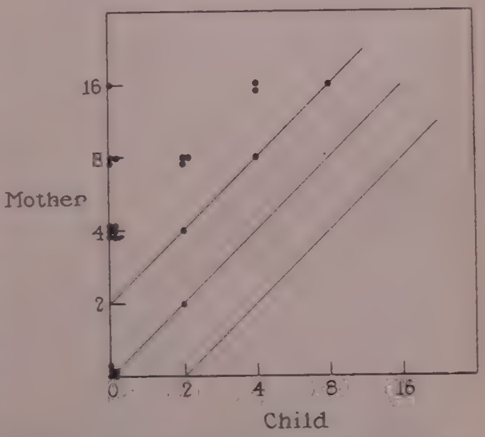

FIG. 3. Comparison of streptococcus MG agglutinin titers in maternal and cord bloods.

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consistent with the earlier findings of Enders and his coworkers as to the incidence of positive reactors among adult urban populations(6).

A comparison of the agglutination-inhibition and complement fixing titers in 20 mothers on whom both tests were done, showed an independence of the level of antibody as measured by each test. That is, some of the sera having the lowest agglutination-inhibition titers had the highest complement fixing titers, and vice versa. This is in agreement with what Florman and Kutch(4) showed to support the contention that the mumps antibodies measured by the complement fixation and agglutination-inhibition tests were distinct and different.

It cannot actually be determined at the present time whether the mumps viral antibodies are smaller in aggregate size than the streptococcus antibodies. However, if they were, it would offer a possible explanation for some of the greater passive resistance of newborn infants to different viral and bacterial infections.

Summary. Quantitative data are presented to show that both the mumps viral complement fixing and agglutination-inhibiting antibodies are transmitted from mother to child without any significant loss in titer. In contrast, titrations for streptococcus MG agglutinins show in most of the instances at least a 4-fold diminution of the maternal titer in the cord blood. There was no difference in results when specimens were from premature or full term infants.

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Growth of *Actinomyces bovis*: Quantitative Cultural Methods. (18998)

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Actinomyces bovis has been grown on a wide variety of media under various cultural conditions. In determining the extent of growth under these cultural conditions, no quantitative technics have been employed. Macroscopic observations with general descriptions of colonial appearance have provided the standards. No methods have been described for using small quantitative inocula. One of the difficulties is that typical rough and intermediate forms(1), when first isolated from parasitic sources in man and animals, appear as non-uniform granules. In addition, many workers transfer undetermined and large numbers of cells with the belief that this technic enhances the possibility of continued growth. Too, the original rough and intermediate forms frequently become smooth after prolonged artificial cultivation.

In order to study the growth requirements of *A. bovis*, it became necessary to develop quantitative methods for determining the size of the inoculum and the amount of growth. Accordingly, experiments were initiated: (a) to preserve the original rough form for an extended period, (b) to obtain maximum growth in liquid media, and (c) to measure the exact amount of growth in various liquid media.

Materials. Three rough isolates from human cases of actinomycosis were used: 43395, which was obtained through the courtesy of Dr. Elizabeth S. Hazen, New York State Department of Health; and AN 11 and AN 14 (from cervico-facial actinomycosis) from Dr. T. Rosebury, College of Physicians and Surgeons, Columbia University. These isolates were grown at pH 7.0 at 37°C in a liquid medium of the following composition: (casitone-yeast extract medium)

casitone (Difco)	15	g
yeast extract (Difco)	5	
glucose	5	
sodium chloride	2.5	
cysteine hydrochloride	.75	
sodium thioglycolate	.3	
water to make 1000 ml		

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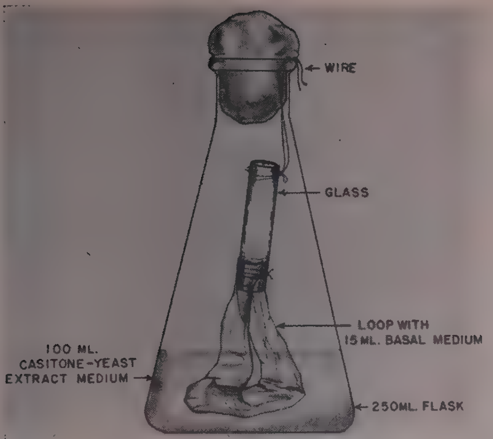

FIG. 1. Diagram of flask with Visking loop for the growth of *A. bovis.*

In the studies employing ridged tubes the casitone and yeast extract were dialyzed before addition to the above medium. Comparable quantities of dialysates were used.

Methods and results. Maintenance of cultures. To maintain the rough form in culture the organism was inoculated into 8-inch loops of Visking tubing containing 15 ml medium suspended in casitone-yeast extract medium (Fig. 1). The original composition of the solution within the Visking loop was sodium chloride, 2.5 g; cysteine hydrochloride, 0.75 g; sodium thioglycolate, 3 g; and water to make 1000 ml (basic salt medium). After 4 to 6 days' growth at 37°C the volume of the internal medium was less than 5 ml, and the growth therein was copious. To insure the availability of the rough forms, the first cultures obtained from the Visking loops were lyophilized.

Determination of standard inoculum. Because of the granular and filamentous nature of the fungus growth, it was impossible to standardize the inoculum by methods based on cell counts. Therefore, 3 to 5 ml of concentrated organisms from a 4- to 6-day-old culture in the Visking tubing were washed thoroughly with 0.85% saline, placed in a 50 ml Erlenmeyer flask with enough 2 mm glass beads to cover the bottom of the flask, and rotated on a Gump shaker for 20 minutes. The resulting suspension was sufficiently homogenous to enable determination of its opacity on a Coleman photo-nephelometer. Serial dilutions of such a cell suspension showed a direct relationship with units on the nephelometer. Measurements were made on the Coleman photo-nephelometer in preference to a photoelectric colorimeter because (a) higher dilutions of the original suspension could be determined directly, and (b) stable reference standards were readily available for instrument calibration. To identify "nephelos units" in terms of concentration of nephelos values of organisms, several different suspensions, prepared as described, were measured and correlated with Kjeldahl nitrogen content (Fig. 2). A direct relationship is indicated between "nephelos units" and amount of cell nitrogen. Therefore, from a number of different suspensions prepared in the manner described above, uniform inocula may be obtained.

Tube culture method for liquid medium. To obtain extensive growth in liquid media, tubes were designed to enable the organism to grow in the optimal zone in relation to the surface when incubated in air. After several trials, the ridged tube, 15 mm × 150 mm (Fig. 3) was found to supply the necessary physical characteristics for growth when inclined at an angle of 30° from the horizontal.

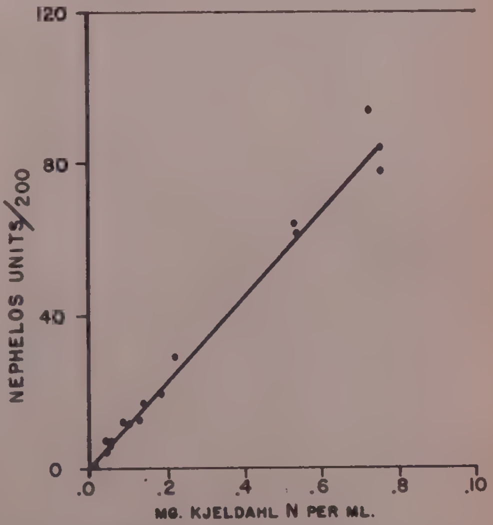

FIG. 2. Relationship between readings of *A. bovis* suspensions (in a Coleman photo-nephelometer) and nitrogen content (Kjeldahl).

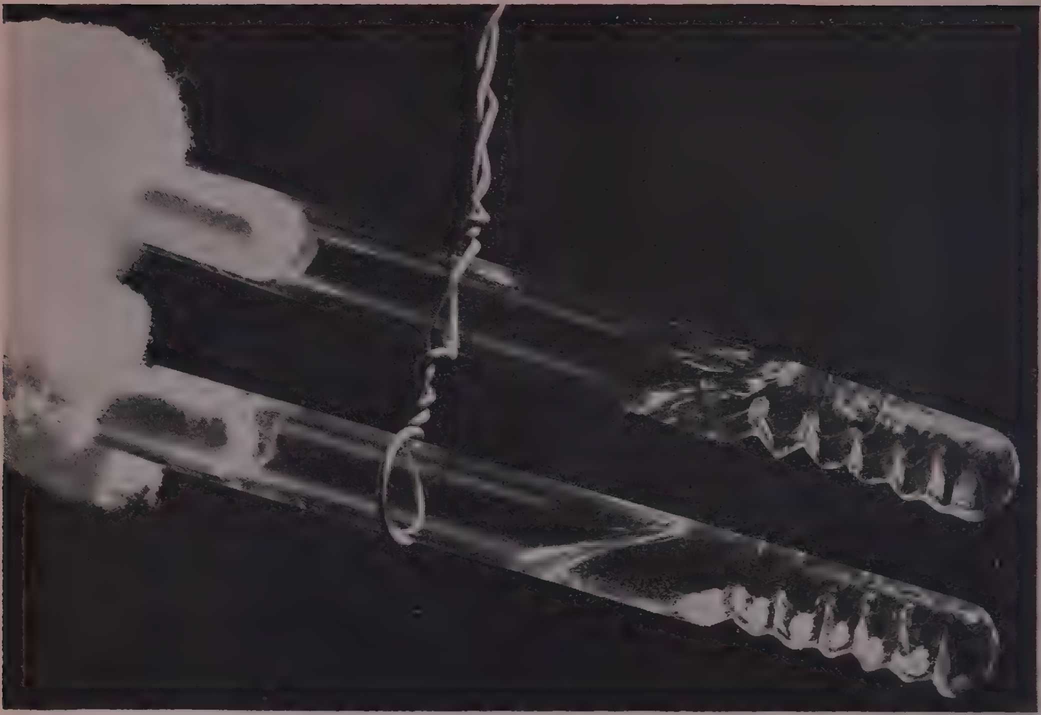

FIG. 3. Photograph of 15 × 150 mm ridged tubes for the growth of *A. bovis.* Upper tube has no growth; lower tube has good growth.

The ridged side, as shown, was placed downward. Plain tubes similarly prepared and inclined showed little or no growth, in comparison with the ridged tubes. In order to insure extensive growth, the ridges were extended to the tube center; shallower ridges gave inferior results. With such ridged tubes, containing 8 ml of the dialyzable medium described, it was found that the minimum standard inoculum for the growth of isolate 43395 corresponded to 0.1 ml of a suspension containing the equivalent of 320 "nephelos units" (equivalent to 1.4 γ Kjeldahl nitrogen). Since the standard suspensions were too concentrated to be measured directly in the nephelometer, appropriate dilutions had to be made to obtain a reading.

Discussion. Growth of *A. bovis* in a liquid medium free of non-dialyzable components provides most advantageous conditions for studies on antigens and growth substances. Also, the use of a liquid medium and the employment of the described methods for accurate determination of the amount of growth offer opportunities for comparison of growth responses under varied experimental conditions.

Summary. (1) Cultural methods are described for obtaining concentrated growth of *A. bovis* in a dialyzable liquid medium. (2) Growth of *A. bovis* has been determined by quantitative technics. (3) A special ridged culture tube has been designed for growing *A. bovis* in a liquid medium under ordinary atmospheric conditions.

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The Frog Test for Staphylococcal Enterotoxin. (18999)

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The recognized need for a more readily available laboratory animal which might satisfactorily substitute for the kitten in the testing of materials believed to contain staphylococcal enterotoxin appeared to be met when Robinton(1,2) reported that frogs could be used for this purpose. She observed that the feeding of staphylococcal culture filtrate to intact frogs induced, after 45 minutes to 7 hours, a specific "spasm" reaction consisting of a series of gaping and swallowing movements performed in a distinctive posture over a 5-10-minute period, climaxed by slow wiping of the face and the sides of the body with fore- and hind-feet respectively. In decerebrate animals the introduction of enterotoxin via the oesophagus into the exposed stomach induced reverse peristalsis either immediately or within 30 minutes depending upon the concentration of the material. Non-enterotoxic filtrates and sterile culture medium had no comparable effects upon intact or decerebrate frogs.

It seemed to us worthwhile to investigate the frog test further, not only because of its possible importance in the study of enterotoxin, but also because it might lend itself to an instructive laboratory exercise for students.

Materials and methods. Seven strains of *Micrococcus pyogenes,* var. *aureus* from various human sources were used. Six strains produced alpha hemolysin and were coagulase positive; 3 of these produced dermonecrotoxin. One strain was coagulase negative and produced no demonstrable alpha hemolysin or dermonecrotoxin. The organisms were inoculated into 125 ml Erlenmeyer flasks containing 35 ml of heart infusion broth with 5% neopeptone and 0.3% agar added, initial pH 7.2. After incubation for 3 days at 37°C in an atmosphere of 30% carbon dioxide (by volume) the cultures were spun for 30 minutes in the cold room at 3600 rpm, using a Size 1 International centrifuge fitted with an angle head. Each culture supernate was decanted and stored separately in a sterile stoppered tube in the cold room. Control supernatant fluid was similarly prepared from uninoculated medium. Portions of the unconcentrated culture supernates and control supernate were concentrated to one fifth of their original volume by evaporation through sterile cellophane sacs placed in front of a fan. Each unconcentrated culture supernate was tested for enterotoxic activity in kittens by the intraperitoneal injection(3) of 2 ml after the first experiment with frogs was completed. Difco Nutrient Gelatin containing 120 g per liter of Bacto-gelatin was used. The honey was a Louisiana product purchased in a local grocery; it was later sampled by us with no ill effects. The frogs used in the early experiments were from Wisconsin (*R. pipiens*) while in later tests Louisiana frogs were employed (the majority *R. clamitans,* a few undoubtedly *R. pipiens sphenocephala*). They were established in the laboratory each in a small museum jar containing a little water and cleaned daily. They were forcibly fed, once a week,* a small ball of ground beef (in the last experiment small pieces of liver were given) and experiments were started 4 hours after feeding. The culture supernates and control media were given orally by medicine dropper and the frogs were watched until it was certain they had swallowed the material. After the liquids were administered the animals were observed steadily for 1 or 2 hours, thereafter at frequent intervals up to 7 or 8 hours while other work was carried on in the same room. Frogs

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3. Dolman, C. E., and Wilson, R. J., *Canad. J. Pub. Health,* 1940, v31, 68.

* As recommended in Carworth Farms Quarterly Letter, No. 11, Oct. 1, 1948.

TABLE I. Results of Administration of Staphylococcal Culture- and Control-Supernates to Kittens and Frogs.

Toxin	Unconcentrated culture supernates	Concentrated culture supernates			
		Intact frogs,* 4/19 and 4/22/51		Decerebrate frogs,* 4/26 and 5/4/51	
	Kittens, 4/3/51	Frog No.	Result	Frog No.	Degree of reverse peristalsis
1	Diarrhea, vomiting	9	Spasm, 6 hr†		
		10	” 3 hr	10	Slight
2	” ”	11	No reaction		
		12	Spasm, 1 hr		
3	No reaction	13	Spasm, 10 min		
		14	No reaction	14	Violent
4	Died	15	No reaction	15	Moderate
		16	Doubtful	16	Slight
5	No reaction	17	No reaction	17	None
		18	” ”		
6	” ”	19	Spasm, 6 hr		
		20	” 5 min	21‡	Strong
7	Slight diarrhea	21	No reaction		
		22	” ”	22	None
Medium Controls	No reaction	23	Spasm, 5 hr	23	Doubtful
		24	” 5½ hr	24	Slight

* Frogs fed meat 4 hr before supernates administered.

† Time elapsed between introduction of supernate and spasm.

‡ This survivor of experiment with toxin No. 7 on 4/22/51 was used to test toxin No. 6 a week later.

that survived these experiments were kept and at a later date, 4 hours after being fed meat, they were decerebrated and the stomachs exposed. The test liquids, in amounts from 0.2-1.0 ml, were introduced via oesophagus directly into their stomachs which were observed for peristalsis. When the experiment lasted for a period of more than 10-15 minutes, physiologic saline was applied to the entire frog at intervals to prevent dehydration.

Results. In a preliminary trial, no reactions were seen in the short period during which unfed frogs were observed after they had been given the unconcentrated culture supernates or control supernate. Twenty-four hours later when food was offered (but not forcibly fed) to the animals, spasms were exhibited by: (1) a frog given an unconcentrated culture supernate demonstrated to produce diarrhea and vomiting in a kitten; (2) a frog given an unconcentrated culture supernate which was negative by kitten test. Two frogs given unconcentrated culture supernates which were enterotoxic for kittens showed no reaction. Under the conditions of this experiment there was no high degree of correlation between the results of the tests in the two species of animals.

Since Robinton had been able by concentrating the filtrates to shorten the period between administration of toxin and the occurence of a reaction, a second group of frogs was tested with the concentrated culture supernates and concentrated control supernate; each supernate was administered to 2 frogs by dropper. The results are summarized in Table I. Frogs 9, 19 and 20 exhibited only short spasms with a few very rapid wiping motions; frogs 10, 12, 13, 20 and control frogs 23 and 24 performed the slowly repeated motions considered most typical. Three of 6 frogs which died within 24 hours were noted to have vomited the meat, practically undigested. The survivors were tested a week or so later in the decerebrate state. Five of the 7 decerebrate frogs tested with concentrated culture supernates and 1 of the 2 given concentrated control supernate showed reverse peristalsis varying from 1 to 8 small upward waves in the pyloric region to a few large waves, the distance traversed varying from about ⅛ inch to one third or one half the

TABLE II. Results of Administration of Concentrated Control Supernates and Other Materials to Frogs.

Material administered	Frog No.	Intact frogs* Date	Intact frogs* Result	Surviving frogs* Date	Surviving frogs* Prepared by	Surviving frogs* Result
Concentrated control supernate	26	6/1	No reaction	6/5	Decerebration	Slight reverse peristalsis
	27		Doubtful		”	” ” ”
	28		Spasm		”	Strong ” ”
	29		No reaction		”	Spontaneous reverse peristalsis upon exposure of stomach after which introduction of material elicited either no response or both upward and downward waves
	30		Spasm	6/6†	”	
	31		Doubtful		”	
Untreated semisolid medium	32	6/19	No reaction	6/26	Curare	Spontaneous reverse peristalsis
	33		Spasm		”	Doubtful ” ”
	34		”		Decerebration	” ” ”
	35		No reaction		”	Spontaneous ” ”
Honey	36		Spasm			
	37		Spasm			
Gelatin	38		No reaction		Curare	Slight ” ”
	39		Spasm		Decerebration	Spontaneous ” ”

* Frogs fed meat 4 hr before material administered.

† Frogs 30 and 31 were decerebrated the following day when their stomachs were empty.

length of the stomach, with the food sometimes being pushed into the upper half of the stomach. Again, no marked correlation with the kitten test for enterotoxin was evident.

Because of the fact that the 2 frogs which had received the concentrated control supernate exhibited spasms, a third group of 6 frogs was tested with the concentrated control supernate only; of these, 2 showed complete spasms and 2 gave doubtful reactions,—gaping and swallowing for a short period or at intervals without the wiping of the face or the abdominal wall. Following decerebration some degree of reverse peristalsis occurred in at least 3 of the frogs. Table II summarizes the observations on this group as well as a fourth group of animals tested with untreated semisolid medium, honey or gelatin. Two of the 4 frogs given untreated semisolid medium, 1 of 2 given gelatin and both of the frogs given honey exhibited spasms. The 2 that received honey vomited within 15 to 30 minutes; both were extremely ill and died. The survivors were tested a week later, with the variation that half of the frogs were injected with 5 units of curare 10 minutes before the stomachs were exposed, instead of being decerebrated, in an attempt to rule out the possibility that reverse peristalsis resulted from the shock of decerebration. However, in frog 32 there was some reversal and contraction of the pylorus before any medium was given; after relaxation of the pylorus, insertion of material was followed only by normal downward waves from the cardia. The other 2 curarized frogs did not show reversal or contraction until after test material was introduced. In decerebrate frogs 35 and 39 strong spontaneous contractions of the pylorus lasting 20 and 40 minutes respectively occurred upon exposure of the stomachs and no reaction was elicited when medium was introduced, possibly because the muscles were still tonically contracted or in the refractory phase.

From Table III which summarizes the findings in frogs tested with materials other than staphylococcal culture supernates, it may be seen that spasms occurred in about half of the intact frogs and that reverse peristalsis resulted in about one third of the decerebrate animals tested with preparations considered to be free of enterotoxin.

Discussion. A satisfactory explanation is not at hand to account for the origin of the spasms and the delay in their onset sometimes observed. Robinton(1), who decapitated frogs at the height of spasm following administration of staphylococcal filtrate, observed reverse peristalsis occurring; in control frogs decapitated at the same time reverse motion of the stomach was not seen. On the other hand, 6 of our decerebrate control frogs

TABLE III. Summary of Experiments with Materials Other Than Staphylococcal Culture Supernates.

	Observation of spasm in							
	Intact frogs				Frogs decerebrate or curarized			
Test material	No. tested	Positive	Doubtful	Negative	No. tested	Positive	Doubtful	Negative
Unconcentrated control supernate	1*			1				
Concentrated control supernate	8	4	2	2	8	4	1	3
Untreated semi-solid medium	4	2		2	4		3	1
Honey	2	2						
Gelatin	2	1		1	2	1		1
Totals	17	9	2	6	14	5	4	5

* Not fed before material given.

showed spontaneous reversal; Mellinger(4) also had observed irregular contractions of the stomach in frogs immediately after pithing. The cause of the reverse peristalsis is unknown.

The consideration that the consistency of the material administered might be of some importance led to the trial of other substances of like property. The concentrated culture supernates and concentrated control supernate were of the viscosity and appearance of maple syrup or honey. The untreated semi-solid medium was not as syrupy as the concentrated supernates, the gelatin even less so, but the honey was quite as glutinous and produced an immediate and prolonged response lasting over a period of hours. These results suggest an action on gastric tone and motility, the reasons for which are not apparent.

Robinton(2) noted a decrease in frequency of spasms occurring in frogs fed toxins during November and complete absence of such responses in December and January. Mellinger (4) found the ability of frogs to vomit upon administration of emetics was absent in January and February, diminished in October and November. His experiments were most successful in the months of June and July and this would seem to imply that there was not at any time 100% response to emetics but a high percentage in summer decreasing to zero in the winter. Our first experiment was performed on March 10th. By April 19th, 50% of the frogs including the controls exhibited spasms and that percentage was maintained through the last experiment on June 19th. Some factor associated with season probably contributes greatly to the higher incidence of spasms during the Spring.

Conclusion. The occurrence of a spasm in frogs fed staphylococcal culture products is either a non-specific response to various substances of an especially viscid nature which is elicited more readily at certain seasons, or is a response at a particular season to some stimulus not yet determined.

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4. Mellinger, C., *Arch. f. d. ges. Physiol.*, 1881, v24, 232.

Beta-Aminoisobutyric Acid, A Possible Factor in Pyrimidine Metabolism.*† (19000)

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The unknown substance previously reported(1) as a major ninhydrin-reacting component in a number of urine chromatograms, particularly in those of patients with malignancy, has now been isolated from the urine of a normal subject(2) and 2 cancer patients and identified as beta-aminoisobutyric acid (BAIB). Preliminary animal experiments indicate that its precursor is a 5-methylpyrimidine.

Experimental. Isolation and identification of beta-aminoisobutyric acid. Urine samples from cancer patients were collected in bottles containing thymol and stored in the freezer. When several specimens containing high concentrations of the unknown compound had been collected from a single patient they were pooled and fractionated in ion-exchange columns. The large variety and high concentrations of extraneous substances in urine made the fractionation more difficult than in the case of protein hydrolysates(3), but ion exchange appears to be the method of choice for separating the small quantity of amino acids in urine from the much larger quantity of strong electrolytes and non-electrolytes. Generally, our best results were obtained as follows: The urine was diluted 2-fold or more, filtered, and passed through a large column (diam. 2-4 cm; ht. 50-70 cm) filled with coarse particles of a cation exchanger of the sulfonic acid type (Amberlite IR-105, Amberlite IR-120, or Dowex 50). The column was washed with water and the amino acids eluted with dilute NH_4OH (0.1 to 0.3 N), the eluent being collected in tubes containing thymol. The band of amino acids occupied only a small portion of this first column and the fronts were often quite irregular (with precipitates sometimes forming local dams which had to be broken by stirring the column) so usually little or no fractionation of the individual amino acids occurred, and all the alkaline ninhydrin-positive fractions were pooled and concentrated *in vacuo* to remove ammonia, and then all the amino acid fractions were combined and diluted for passage through a second column. The second column (diam. 1-2 cm, ht. 40-60 cm) contained 60-80 mesh particles of an anion exchanger of the strong base type (Amberlite IRA-400). The pooled fractions from the first column were added, the column was washed with water, and the amino acids were displaced with 0.5 N NH_4OH or 0.16 N acetic acid. (Acid elution sometimes caused formation of gas bubbles, presumably CO_2, in such quantity as to interfere with the flow and necessitate their removal by occasional stirring of the column). Usually some separation of the various amino acids occurred at this stage and only the fractions found chromatographically to contain significant concentrations of the unknown compound were pooled, concentrated *in vacuo*, and diluted. Final ion exchange fractionation was carried out in a column 3-10 mm in diameter and 40-60 cm high, filled with sulfonic acid-type resin of 80-100 mesh. The selected eluate of the previous column, a water wash, and

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† Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

1. Fink, K., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 692.

2. Crumpler, H. R., Dent, C. E., Harris, H., and Westall, R. G., *Nature*, 1951, v167, 307.

3. Partridge, S. M., and Westall, R., *Biochem. J.*, 1949, v44, 418.

dilute ammonia were passed through at a rate of 1 ml per minute, or less, and the eluate was collected in small fractions.

The best ion exchange fractions contained the unknown compound as the only ninhydrin-reacting component, but the usual procedures failed to produce crystals, and concentration and chromatography revealed the presence of colored and fluorescent substances. Accordingly, some of the best fractions were streaked across large sheets of washed filter paper, chromatographed in butanol, acetic acid, and water (2:1:1), and the areas containing the ninhydrin reacting substance were isolated and eluted as previously described(1) or by Soxhlet extraction with methanol.

A solution of the unknown compound prepared from the urine of one cancer patient by ion exchange and chromatography yielded 67 mg of a crystalline benzenesulfonyl derivative when subjected to the Schotten-Baumann procedure(4). This derivative, when recrystallized from water or toluene (0.2 ml per 10 mg), melted at 83.0 to 83.6°C[‡] and yielded equivalent weight values of 254, 247, and 247. (Equivalent weight calculated for the benzenesulfonyl derivative of a 4-carbon, mono-amino, mono-carboxylic acid 243.3).

Analysis[§]—$C_{10}H_{13}NSO_4$.
Calculated: C 49.36, H 5.39, N 5.76
Found: C 49.49, H 5.63, N 5.68

Hydrolysis of a small portion of the derivative overnight at 100°C in 12 N HCl yielded a ninhydrin-reacting compound with the original chromatographic properties. The isolated substance could be distinguished from the available 4-carbon mono-amino, mono-carboxylic acids, alpha-amino-n-butyric acid, alpha-aminoisobutyric acid, and gamma-amino-n-butyric acid by its chromatographic properties and reaction to cupric ion.[¶] Its stability to acid hydrolysis served to distinguish it from beta-amino-n-butyric acid(5), and comparison of the chromatographic behavior of glycine and sarcosine appeared to rule out N-methyl alanine, N-methyl beta-alanine, N-dimethyl glycine, and N-ethyl glycine as possible structures for the unknown. The remaining isomer, beta-aminoisobutyric acid, was synthesized following the procedure of Clemo and Melrose(6) by HCl hydrolysis of the 5-methyl-4,5-dihydrouracil obtained from the condensation of urea and ethyl methacrylate. The synthetic amino acid was chromatographically indistinguishable from the urinary compound and gave the same reaction to copper. A benzenesulfonyl derivative of the synthetic BAIB, prepared as before, melted at 80-83°C, and a mixture of the synthetic and natural derivatives melted at 79-82.5°C.

The "best fractions" from the urine of a second cancer patient yielded about 15 mg of clean crystals of the free amino acid after the fractions were evaporated, dissolved in hot butanol and twice recrystallized. The isolated compound melted at 181-184°C, a synthetic sample of BAIB prepared by Pollack(7)[‖] melted at 177-178°C, and a mixture of the natural and synthetic compounds melted at 163-178°C. The infra-red absorption spectra of the natural and synthetic compounds showed only slight differences, similar to those shown by the spectra of *l* and *dl* leucine,[††] and as the two preparations also appeared to be identical in their reaction to copper and their chromatographic behavior in various solvents, it was assumed that the melting point variation was due to optical isomerism in the natural BAIB. This assumption appears to

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4. Keston, A. S., Udenfriend, S., and Cannan, R. K., *J. Am. Chem. Soc.*, 1949, v71, 254.

[‡] All melting points corrected.

[§] Elek Micro Analytical Laboratories, Los Angeles.

[¶] The previous paper(1) failed to make clear that it was a *variation* of the Crumpler and Dent(5) copper technic, with the amino acids remaining in the copper-brushed area of a one-dimensional chromatogram, in which ninhydrin gave a peculiar color reaction with the unknown compound. The two-dimensional technic described by Crumpler and Dent results in the usual color reaction.

5. Crumpler, H. R., and Dent, C. E., *Nature,* 1949, v164, 441.

6. Clemo, G. R., and Melrose, T. A., *J. Chem. Soc.,* 1942, 424.

7. Pollack, M., *J. Am. Chem. Soc.,* 1943, v65, 1335.

[‖] The authors are indebted to Dr. L. J. Reed who sent us a sample of Pollack's synthetic BAIB.

[††] We are indebted to Dr. R. E. Nusbaum of the U.C.L.A. Atomic Energy Project for measuring and studying the infra-red absorption spectra.

have been verified by the recent publication of Crumpler, Dent, Harris, and Westall(2), whose isolated BAIB was laevorotatory, melted at 183-184°C, and required racemization before melting point and X-ray diffraction data showed its identity with Pollack's (7) synthetic BAIB.

*Clinical studies.*** First morning urine specimens were collected in bottles containing thymol, an aliquot was stored at —20°C for possible rechecking, and the remainder stored at 5°C until the chromatograms were set up, which was rarely more than 2 hours after the specimen was voided. Duplicate two-dimensional filter paper chromatograms were prepared from 25 μl aliquots of the fresh urine specimens, one with known amino acids added as markers, using water-saturated phenol as the first solvent and the upper phase from a mixture of tertiary butanol, secondary butanol, and water (1:5:5.6) as the second solvent. The density of the ninhydrin-developed spots was determined with a photoelectric cell, and the urinary concentration of the various amino acids then estimated from standard curves prepared from similar chromatograms containing a known number of micromoles of the various amino acids. The standard curves could be reproduced within about ±20%, and addition of known amounts of various amino acids to urine indicated that much of our data on urinary amino acids also falls within this range of accuracy. Overlapping, streaking, or distortion of the spots on urine chromatograms can increase the error markedly, however, and the routine urinary data, particularly as regards amino acids present in very high or very low concentrations, are considered as potentially in error by a factor of two or more.

Data on BAIB levels in urine specimens from a group of 140 apparently healthy subjects (physicians, nurses, laboratory personnel, and members of an Air Force National Guard unit) are shown in Table I. The variability in concentration of the more common urinary amino acids and in the chromatographic development is to some extent compensated for by subdividing the group according to the ratio of BAIB to alanine shown by each chromatogram. In 63% of the group studied BAIB was not detectable, indicating urinary levels less than about 0.15 micromole per liter, the ninhydrin test being only about half as sensitive for BAIB as for most of the simple alpha-amino acids. In 29% of the cases BAIB was detectable but showed concentrations less than twice those of alanine. Eight individuals of the 140 showed BAIB levels 2 to 3 times their alanine levels, and 3 subjects showed still higher BAIB/alanine ratios.

TABLE I. Urinary Excretion of beta-Aminoisobutyric Acid by 140 Apparently Normal Humans.

No. of cases	BAIB/alanine ratio	Avg BAIB conc., millimoles/liter
88	BAIB not detectable	<.15
41	.3–1.9	.3
8	2–3	.4
1	5	2.5
2	12–13	3

Medical histories of individuals included in the control group were not requested, but the individual who showed the highest BAIB concentration was at first dubious about contributing a control specimen because of a family history of malignancy. This individual has remained in good health during the 1½ year period since the first observation. One of the early members of our control group has regularly shown a high BAIB level for the past 2 years (about one millimole per liter, 5 times his alanine level), but was omitted from the control group reported here since he recently noted the appearance on his leg of a small, rapidly growing nodule which was subsequently excised and diagnosed as a primary fibrosarcoma. Several individuals in the normal group were checked at infrequent intervals for periods up to 2½ years and were found to show little variation in their BAIB excretion. In day-to-day studies of patients with neoplastic disease, on the other hand, extreme variations in the urinary BAIB level have occasionally been noted, and the level

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** The authors are indebted to several members of the staff of the Veterans Administration Hospital, and especially to Dr. Philip M. West and Dr. Frank W. Ellis of the Neoplastic Disease Section, for their cooperation in the clinical studies.

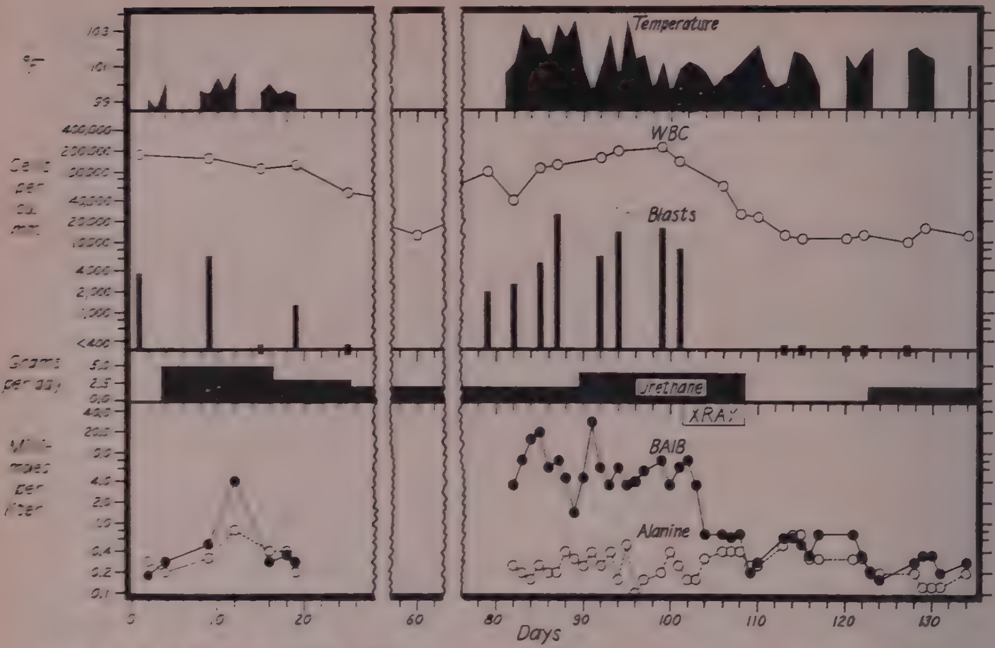

FIG. 1. Data in case of chronic myelogenous leukemia. Note that the blood cell counts and the urinary levels of alanine and BAIB (beta-aminoisobutyric acid) are plotted on logarithmic scales while the body temperature and urethane dosage are on arithmetic scales. The patient was acutely ill and bedridden during period from about 81-105 days but felt well and was ambulatory during the remainder of the observation periods.

seems to be affected by therapeutic regimes and the clinical state of the disease. The factors responsible for the variations are not clear, however, and although over a thousand urine specimens from cases of malignancy have been analyzed with duplicate 2-dimensional chromatograms, it appears that an intensive study of the relevant case histories as well as the collection of a larger quantity of data will be required before it can be accurately determined whether BAIB is significantly related to any given type of neoplasm or other disease.

The variability of BAIB excretion seen in some patients is illustrated in Fig. 1, which shows a portion of the clinical data obtained during 2 flareups of a case of chronic myelogenous leukemia. A marked elevation of urinary BAIB was found in only one specimen (day No. 12) during the first of the two episodes shown, and it was accompanied by a moderate elevation of other urinary amino acids. The patient had been hospitalized on the basis of his blood count at the beginning of this period (note that both the blood counts and the urinary amino acid levels are plotted on logarithmic scales) but he continued to feel well, was allowed to leave the hospital on weekends, and after about three weeks was discharged to return to his work and continue urethane therapy at home. His blood picture gradually improved, but on the 81st day an exacerbation of the leukemic process occurred and the patient was acutely ill for about three weeks, with a very high total white blood cell count, a high proportion of immature cells, fever, and a greatly enlarged spleen. Following an increase in urethane dosage and X-ray to the spleen the patient again became ambulatory, showed a drop in white count and blast cells, gained 10 pounds in 2 weeks, was allowed to go home on weekends, and finally was discharged with instructions to continue urethane therapy. During the acute phase of this second episode the urinary BAIB reached levels as high as 30 millimoles per

TABLE II. Urinary Excretion of beta-Aminoisobutyric Acid, Alanine, and Leucines by Rat No. 1 Under Various Dietary Regimes.

Day	Diet	Urinary concentration (μM/20 ml)			Urine vol (ml)	Rat wt (g)
		BAIB*	Alanine	Leucines		
1	chow	0	2	0	16	250 (est.)
2	40% DNA†	20	24	6	19‡	
3 day	”	120	2	2	2	
3 night	chow	2	2	1	17	
4	”	2	3	1	27	
5	”	0	3	2	10	
6	”	0	2	2	22	205
7	8% thymine§	4	2	1	32	
8	”	3	2	1	34	
9	chow	5	4	2	17	
10	”	0	3	2	18	
11	”	0	2	1	13	215
12	”	0	2	2	14	
13	”	0	2	2	14	
14	10% DNA	3	2	1	16	231
15	”	3	2	1	20	237
16	”	6	2	1	26	236
17	”	5	4	1	25	230
18	”	3	2	1	25	232
19	”	3	2	1	20	241
20	chow	6	2	2	14	229
21	”	0	2	1	16	252
22	”	0	1	1	14	251

* The chromatogram spot reported in this column has been identified as BAIB (beta Aminoisobutyric acid) with reasonable certainty in the case of the 40% DNA diet, and it is probable, but less certain, that the new spot which appeared during the thymine and 10% DNA feedings was also BAIB.

† Desoxyribonucleic acid from sperm (Nutritional Biochemicals Corp., Cleveland, Ohio). The rat consumed 4.2 g at the 40% level and 8.3 g at the 10% level.

‡ Diarrhea, sample contaminated with feces.

§ Rat consumed 1 g of thymine.

liter (rechecked for accuracy with smaller aliquots), about a 200-fold increase over some previously determined levels, and then fell again as the patient's clinical condition improved. The great variation in BAIB excretion was not caused by a generalized aminoaciduria, for the levels of other regularly detectable amino acids were found to show only about 3 to 10-fold variations in the urine specimens of this patient.

Animal experiments. Adult male albino rats were placed in metabolism cages and fed finely ground purina dog chow for a day or two to permit collection of control urine specimens. During an experimental period the dry test substance was thoroughly mixed with powdered chow. The urine was collected under toluene and chromatograms were prepared as in the clinical studies. The excretion levels of BAIB in the urine of two rats subjected to various dietary regimes are shown in Tables II and III, and data on the excretion of alanine and the leucines are included for comparison. Neither rat excreted detectable amounts of BAIB during the preliminary basal diet period. The 40% desoxyribonucleic acid (DNA) diet resulted in a marked loss in weight, a short period of diarrhea, a brief and generally small rise in the concentration of several urinary amino acids, and the appearance of a very high concentration of BAIB. The 40% ribonucleic acid (RNA) diet likewise caused a drop in body weight and production of very soft (but not liquid) feces, but it failed to produced notable changes in amino acid excretion. A 10% DNA diet was well tolerated by the rat for several days and chromatograms prepared during this period showed a rather light spot in the position taken by BAIB. The 8% thymine diet produced a similar spot, but not as dark as would have been expected from feeding DNA containing an equivalent quantity of thymine.

TABLE III. Urinary Excretion of beta-Aminoisobutyric Acid, Alanine, and Leucines by Rat No. 2 Under Various Dietary Regimes.

		Urinary concentration (μM/20 ml)			Urine	Rat
Day	Diet	BAIB*	Alanine	Leucines	vol (ml)	wt (g)
1	chow	0	2	2	26	300
2	40% DNA†	4	7	8	22	
3	”	40	2	2	22	
4	”	70	5	2	28‡	
5	chow	10	4	2	31	267
6	”	0	4	2	21	
7	”	0	5	2	29	
8	”	0	3	2	21	305
9	40% RNA§	0	3	1	32	283
10	”	0	3	1	18	269
11	”	0	2	1	10	274
12	chow	0	5	2	22	
13	”	0	3	2	37	304
14	”	0	3	2	30	317
15	”	0	2	2	18	313

* beta-Aminoisobutyric acid.

† Desoxyribonucleic acid from sperm (Nutritional Biochemicals Corp., Cleveland, Ohio). Rat consumed 8.2 g.

‡ Diarrhea, sample contaminated with feces.

§ Ribonucleic acid (Nutritional Biochemicals Corp., Cleveland, O.). Rat consumed 7.7 g.

Rat urines generally yield poor chromatograms, and the area of the chromatograms occupied by BAIB is potentially a crowded one(1), but there is very little doubt that the intense spot produced by the 40% DNA diet actually was BAIB, for its chromatographic behavior in a number of solvents, its stability to acid hydrolysis, and its reaction to copper appeared to be identical with that of the BAIB isolated from urine of humans with neoplastic disease. Although the 8% thymine and 10% DNA diets each produced an acid-stable compound which gave a spot in the chromatographic position of BAIB, and these spots are listed as BAIB in Table II, the concentrations in these cases were so low that a partial purification will be required before extensive chromatographic and chemical tests can establish the identity of the spots beyond reasonable doubt.

Discussion. There appears to be no doubt that many human subjects do excrete BAIB and that some of them excrete a greater amount of it than of any other free amino acid. In addition to the data presented in this paper, Crumpler *et al.*(2) have reported that about 5% of their large group of normal subjects showed consistently high urinary levels of BAIB and that a study on relatives of some of these indicated the involvement of a genetic factor. Some of the available data suggest that BAIB may be related to the cancer process, but a determination of whether the suggested relationship is real and of metabolic significance will require further study.

A "blackboard survey" of conceivable BAIB precursors pointed to the 5-methylpyrimidines as likely possibilities. In preliminary animal experiments a diet high in 5-methylpyrimidine—containing DNA caused BAIB excretion and appeared to support the hypothesis, especially since a similar diet in which the DNA was replaced by RNA failed to show the effect and thus served as a control which tended to rule out some possible complicating factors such as an adenine effect on the kidney(8). A diet high in free thymine caused excretion of a substance tentatively identified as BAIB, but at a lower concentration than might have been expected on the basis of the probable combined thymine content of the DNA diet. It is possible that the free thymine was poorly absorbed or inefficiently catabolized, and isotopic studies or feeding experiments with thymidine, 5-methylcytosine(9,10), and other compounds may

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8. Bendich, A., Brown, G. B., Philips, F. S., and Thiersch, J. B., *J. Biol. Chem.*, 1950, v183, 267.

9. Wyatt, G. R., *Nature*, 1950, v166, 237.

clarify the point. No evidence has been obtained to indicate which of several conceivable biochemical pathways might be involved in the formation of BAIB from a 5-methylpyrimidine.

The hypothesis(2) of a genetically determined error in kidney resorption seems logical as an explanation of the high BAIB levels in some normal individuals, but it does not appear to be consistent with clinical data showing marked lowering of the BAIB excretion following therapeutic regimes more likely to injure than to improve kidney function. The studies described suggest, as one alternative hypothesis, that a high BAIB level might indicate an abnormal rate or type of 5-methylpyrimidine catabolism.

Summary. (1) A compound frequently noted in a chromatographic study of urinary amino acids has now been isolated from the urines of two cancer patients and identified as beta-aminoisobutyric acid (BAIB). (2) In a group of 140 apparently healthy subjects, 52 individuals showed detectable urinary levels of BAIB (>0.15 millimole per liter), and three of these showed concentrations over 1 millimole per liter. Individual control subjects studied at intervals for periods up to 2½ years showed little fluctuation in BAIB excretion. (3) In individual patients with neoplastic disease, the urinary BAIB level occasionally varied markedly and appeared to bear some relationship to the neoplastic process. Such variations are illustrated by a report of a case of chronic myelogenous leukemia. (4) Theoretical considerations pointed to a 5-methylpyrimidine as a likely precursor of BAIB, and evidence favoring this view was obtained by rat experiments in which BAIB excretion was produced by a diet containing large amounts of desoxyribonucleic acid but not by one similarly enriched in ribonucleic acid.

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10. Johnson, T. B., and Coghill, R. D., *J. Am. Chem. Soc.*, 1925, v47, 2838.

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Effect of Cortisone on the Mast Cells of the Rat. (19001)

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Recent observations have stressed the important role played by cortical hormones, especially cortisone, in the collagen diseases and in the biochemistry of the mesenchymal changes in dermal connective tissue both in man and in laboratory animals under normal and pathological conditions. These changes concern both the fibrillar and cellular constituents of the connective tissue and the homogeneous interfibrillar ground substance. There are two prevalent opinions concerning the physiological significance of the mast cells: some authors believe that these cells produce the hyaluronic acid of the ground substance; according to others the mast cells produce heparin.

In view of the evident changes caused by cortisone on acid muco-polysaccharides (hyaluronic acid) of the tissues, we wished to ascertain whether these histochemical and biochemical changes were related to the morphology of the mast cells.

Material. For this purpose, 36 unselected adult male rats, fed on a fixed and balanced standard diet, were divided in groups of 6 animals. Three groups were injected subcutaneously with cortisone ("Ciba") 10-5-1 mg/day per animal for 3-20-70 days respectively. For the same period 3 groups of control animals were given similar doses of physiological saline alone. At the end of experiment all animals were killed with a sharp blow on the head. Specimens were taken from the skin of the neck and from the muscles of the thigh and myocardium; these were fixed in Carnoy fluid, cut in series, and stained with an aqueous

TABLE I. Effect of Cortisone on the Mast Cell Counts in the Skin, Muscle and Myocardium of the Rat.

Group	Dosage	No. of mast cells per mm² of tissue		
		Skin	Muscle	Myocardium
Controls	saline 3 days	68.13 ± .57*	5.55 ± .50	4.41 ± 1.24
	20	70.64 ± 2.70		
	70	81.13 ± 2.30	7.09 ± .23	3.73 ± .34
Cortisone	10 mg 3	30.90 ± 2.16	1.50 ± .39	1.22 ± .60
	5 mg 20	30.58 ± 4.50		
	1 mg 70	25.31 ± 5.20	3.30 ± .15	.59 ± .10

* Stand. error calculated following the formula $\sqrt{\frac{S\,x^2}{n(n-1)}}$.

0.05% toluidin blue solution for 18 to 20 hours according to Bunting(1). Microscopical examination of each specimen was carried out and the mast cells were counted. For this purpose we used a magnification of 200 diameters, counting the mast cells in 300 microscopic fields in each specimen and in each animal. The values reported in Table I represent the calculated number of mast cells per mm² of tissue examined. Hyaluronic acid in the connective tissue was identified by means of bull testis hyaluronidase and subsequent staining with periodic acid-leucofuchsin according to Hotchkiss(2); the enzyme was employed in a concentration of 15 turbidity reducing units per cc in 0.3% NaCl solution at 37°C per 18 hours.

Results. Cortisone treated rats showed a marked fall of the body weight, a decrease of the eosinophil and lymphocyte count in the peripheral blood, and atrophic changes of the spleen, thymus, and bone marrow as well as a rise of the liver glycogen. In these rats the skin was atrophied and presented a clear decrease in the dermal hyaluronic acid; fibroblasts were scanty, and the fibrils reduced in number. The deeper layers of the corium showed an evident reduction of the loose connective tissue. In all the experimental series the mast cells were clearly reduced in number (see table) and in size, and the granules were similarly affected; many of the cells were disintegrated and the granules dispersed in the ground substance. As in normal animals, these cells showed mainly a perivascular distribution. The metachromatic substance of the granules was refractory to the action of hyaluronidase, in contrast with the ground substance of the connective tissue.

The examination of the muscular tissue and myocardium gave similar results: metachromatically stainable material had practically disappeared from the connective interfibrillar tissue and the mast cells were markedly decreased in number (see table).

Discussion. It has been claimed that cortisone and ACTH reduce the hyaluronic acid content of the mesenchymal tissues both in normal and pathological conditions(3,4). On the other hand it has been demonstrated that the administration of cortisone in man is rapidly followed by a rise of the circulating heparin-like substances(5,6).

It is very probable that both these changes are intimately correlated with the inhibitory effect of cortisone and the mast cells. In our experiments the decrease in number of these elements was very marked and at the same time a loss of the metachromatically staining material of the connective tissue could be observed. Similar results were obtained by Asboe-Hansen(7) in skin biopsies from pa-

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2. Hotchkiss, R. C., *Arch. Biochem.*, 1948, v16, 131.

3. Asboe-Hansen, G., *Bull. d'hist. appl. a la physiol.*, 1950, v27, 5.

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tients treated with ACTH; this author was of the opinion that hyaluronic acid is a product of the mast cells and that these cells are the peripheral transmitters of the cortisone hormonal action.

In other experiments, we have been able to show that the inhibitory effect of cortisone is well marked on all the cellular constituents of the connective tissue: fibroblast, reticulo-endothelial system, plasma cells(8,9). The reduction in number of mast cells may be regarded as a partial manifestation of this general inhibitory effect of the hormone. In other unpublished experiments we have also observed that in agreement with others(10), the hormone, also when locally applied decreases the mast cells and other cellular constituents in the dermal tissue. It is very likely therefore that the action of cortisone is more pharmacological than hormonal in nature.

Summary. Adult rats, injected with cortisone in various doses and for a different period, constantly showed a marked reduction in the mast cell counts of the dermal, muscular and myocardial tissue, together with a strong decrease of hyaluronic acid of the connective tissue. Similar results were observed in the skin when the hormone was applied locally. It is pointed out that a close correlation exists between the mast cells and the hyaluronic acid and that cortisone acts as an inhibitory factor on these cells, just as on the other cellular constituents of the mesenchymal tissues.

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Effect of Soybean Sterols in the Diet on Plasma and Liver Cholesterol in Chicks. (19002)

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It is generally agreed that the absorption of sterols from the intestinal tract is highly specific. Cholesterol is readily absorbed by animals, especially in the presence of fat. Schoenheimer and associates(1-3) and others (4) have shown that a number of plant sterols are not absorbed.

The experiments described in this report were suggested by the author's observation that simultaneous addition of soybean sterols and cholesterol to the diet did not produce the rise in plasma cholesterol which was obtained when cholesterol alone or in oil was added to the chicks' diet.

Animals. The experimental animals were 14-day-old single comb White Leghorn chicks that had been maintained on a standard stock diet from the time of hatching. In the preliminary experiments (Fig. 1) chicks of both sexes were used. In the later experiments (Tables I and II) only males were employed.

Diets. The basal diet used in these experiments was the same as that previously reported by Peterson(5). Additions of cholesterol, soy sterols* and cottonseed oil were made by substitution for a like amount of

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2. Behring, H. v., and Schoenheimer, R., *Z. physiol. Chem.*, 1930, v192, 97.

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4. Sperry, W. M., and Bergmann, W., *J. Biol. Chem.*, 1937, v119, 171.

5. Peterson, D. W., *J. Nutrition*, 1950, v42, 597.

* Mixed Soy Sterols containing a minimum of 95% sterols, principally sitosterols and stigmasterol, were supplied through the courtesy of Mr. C. N. Behrndt of Distillation Products Industries.

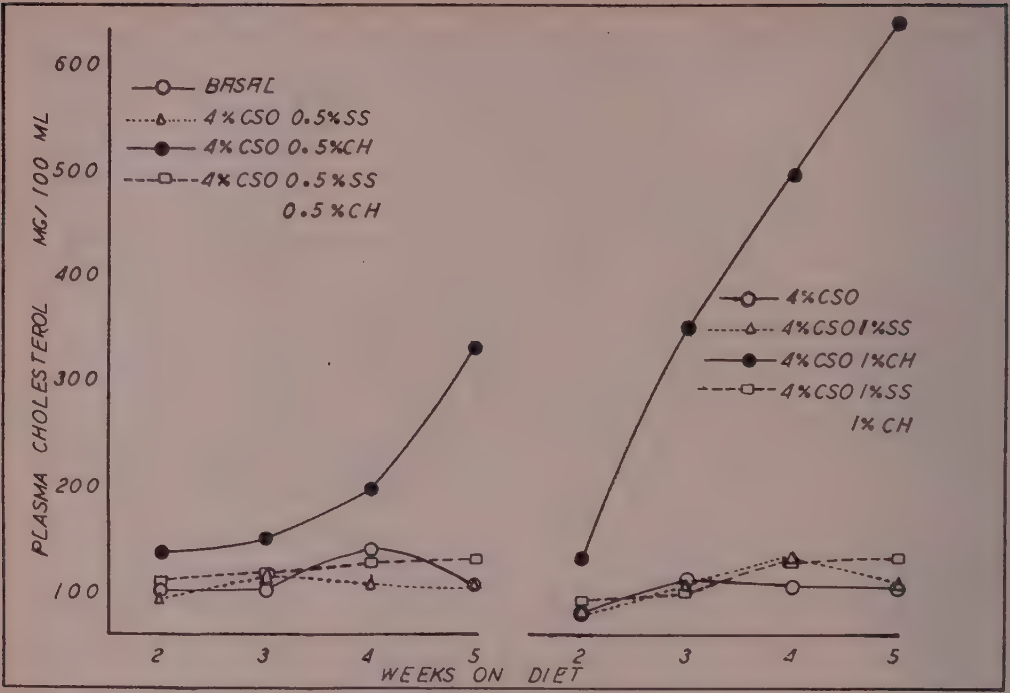

FIG. 1 and 2. Effect of soy sterols on plasma levels of cholesterol. CSO = cottonseed oil; SS = soy sterols; CH = cholesterol.

barley in the basal diet. Cholesterol and soy sterols or mixtures of these were dissolved by warming in cottonseed oil.† The thick paste which resulted on cooling was then mixed in the diet.

Analytical. Samples of heparinized plasma were analyzed for total cholesterol by the method of Schoenheimer and Sperry as modified by Sperry and Webb(6). Technics of sampling varied as indicated below. In the later experiments, livers were also analyzed for total cholesterol. They were taken at autopsy, weighed and frozen immediately. Weighed samples were later homogenized with ground pyrex glass using a mortar and pestle. The homogenate was then transferred to an Erlenmeyer flask with the aid of wet filter paper and covered with 95% ethyl alcohol. The alcohol was removed by evaporation on a steam bath and the residue extracted with several portions of a 1:1 mixture of absolute alcohol and acetone until the extract no longer gave a Liebermann-Burchard reaction. These extracts were then diluted to volume and aliquot samples analyzed by the Schoenheimer-Sperry procedure(6). Moisture determinations were made on each liver sample by drying at 100°C to constant weight.

Results and discussion. Several preliminary experiments showed that the addition of a mixture of soy sterols and cholesterol in cottonseed oil to the diet resulted in considerably lower levels of cholesterol in the plasma than the addition of cholesterol in cottonseed oil. Typical results of such an experiment are shown in Fig. 1.

Feeding cottonseed oil or a mixture of cottonseed oil and soy sterols in the diet did not increase total plasma cholesterol beyond the levels observed in chicks fed the basal diet alone.

Feeding of cholesterol and cottonseed oil produced the typical rise in plasma cholesterol

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† Wesson Oil.

6. Sperry, W. M., and Webb, M., *J. Biol. Chem.*, 1950, v187, 97.

TABLE I. Plasma and Liver Levels of Cholesterol in Male Chicks Fed Various Diets.

	3 wk on diet		4 wk on diet			
					Liver total cholesterol	
Diet	No. of birds	Plasma total cholesterol, mean ± S.D., mg/100 ml	No. of birds	Plasma total cholesterol, mean ± S.D., mg/100 ml	Wet wt, mean ± S.D., %	Dry wt, mean ± S.D., %
S-1 Basal	7	86 ± 34	6	91 ± 14	.295 ± .025	1.12 ± .15
S-2 Basal + 4% cottonseed oil	6	75 ± 9	6	85 ± 8	.275 ± .026	1.01 ± .12
S-3 Basal + 4% cottonseed oil + 1% cholesterol	6	456 ± 147	7	553 ± 95	1.40 ± .238	5.21 ± .78
S-4 Basal + 4% cottonseed oil + 1% cholesterol + 1% soy sterols	7	112 ± 13	7	109 ± 19	.437 ± .099	1.69 ± .39
S-5* Basal + 4% cottonseed oil + 1% cholesterol + 1% soy sterols	7	130 ± 28	7	133 ± 35	.554 ± .116	1.99 ± .41
S-6 Basal + 4% cottonseed oil + 1% soy sitosterols + 1% cholesterol	7	136 ± 21	7	109 ± 9	.462 ± .104	1.65 ± .34

* Cholesterol and soy sterols added separately to diet.

TABLE II. Plasma and Liver Cholesterol Values for Male Birds Fed for 10 Weeks.

	Plasma total cholesterol (composite samples from 3 chicks)				Liver total cholesterol	
	Weeks on diet (mg/100 ml)				10 wk on diet	
Diet	4	6	8	10	Wet wt, %	Dry wt, %
No. 1 Basal + 4.5% cottonseed oil	82	78	84	108	.309	.970
	78	82	72	88	.330	1
	82	95	83	121	.304	.937
Avg	81	85	80	106	.314	.969
No. 2 Basal + 4.5% cottonseed oil + 1% cholesterol	542	517	719	766	2.48	8.37
	542	554	1503	534	2.49	8.07
	876	1138	576	1526	2.88	12
Avg	653	736	933	942	2.61	9.48
No. 3 Basal + 4.5% cottonseed oil + 1% cholesterol + 1.3% soy sterols	108	121	138	192	.407*	1.28*
	124	121	124	138	.552	1.75
	139	131	121	143	.572	1.72
Avg	124	124	128	158	.510	1.62

* Values averaged from 2 livers. All other values are averages of determinations on 3 individual livers.

obtained with continued cholesterol feeding(7). This rise was more rapid when cholesterol was fed as 1% of the diet than 0.5%, however there was a significant rise with 0.5% cholesterol. Addition of the soy sterols to the cottonseed oil-cholesterol diet prevented the rise in plasma cholesterol over the 5-week feeding period (Fig. 1). In this experiment cottonseed oil was employed at the 4% level. Cholesterol and soy sterols were used at 2 levels: 1% and 0.5% of the diet. Each week heparinized blood samples were taken by heart puncture from chicks which had been anesthetized with nembutal. It was necessary to sacrifice the chicks in order that

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7. Horlick, L., and Katz, L. N., *Am. Heart J.*, 1949, v38, 336.

sufficiently large samples could be obtained for individual analyses. Each point on the curve represents an average value for 4 chicks.

The results of an experiment using male chicks are shown in Table I. Blood samples were taken by heart puncture when the chicks were sacrificed. Half of the chicks were sacrificed after the third week on the diets; the remainder after the fourth week on the diets when liver and blood samples were taken for analysis. The groups fed diets S-1 to S-4 present results essentially the same as those previously obtained. Diet S-5 was identical in composition with diet S-4 with the exception of the method of addition of the fat and sterols. Here the cholesterol was dissolved in half the cottonseed oil and added to the diet. Soy sterols were dissolved in the remainder of the cottonseed oil and added to the diet. The purpose of this procedure was to eliminate the possibility that the results obtained might be due to changes occurring while heating the mixture of soy sterols and cholesterol. It can be seen from Table I that plasma cholesterol remained low on this diet (S-5) regardless of the method of mixing the sterols.

Diet S-6 contained 1% of a purified sitosterol fraction (m.p. 128-130°C) of soybean sterols obtained from a commercial source. The results obtained with the diet containing this fraction were similar to those obtained with the mixed soy sterols. Further work using purified sterols will be necessary in order to determine whether the effect obtained is a characteristic of plant sterols in general or whether it is a property of sitosterols in particular.

The values for total liver cholesterol are given for both the wet and dry basis (Table I). Feeding of cholesterol and cottonseed oil produced the usual deposition of cholesterol in the liver. Livers of these birds were grossly fatty in appearance. Livers of birds on the control diets (S-1 and S-2) and of the birds on the phytosterol-cholesterol diets were normal in appearance. Livers of the birds on the soy sterols diet, although slightly higher in cholesterol than the controls, were much lower than those fed cholesterol alone. This was possibly due to decreased absorption of cholesterol owing to interference by one or more constituents of the mixed sterols.

The results shown in Table II show plasma and liver cholesterol levels for male chicks fed 3 diets over a period of 10 weeks. All diets contained 4.5% added cottonseed oil, diet 2 contained in addition 1% cholesterol, diet 3 1% added cholesterol and 1.3% added soy sterols. Chicks were anesthetized with nembutal and heparinized blood samples were taken from the wing veins every 2 weeks beginning with the fourth week on diet. Analyses for total cholesterol were made on composite samples from 3 chicks. The chicks were sacrificed after 10 weeks on the diets and liver samples analyzed for total cholesterol. The values in Table II represent averages of determinations on 9 individual livers for Diets 1 and 2 and 8 livers for Diet 3. These data show again the pronounced rise in total plasma cholesterol of the group fed cholesterol and cottonseed oil. Plasma cholesterol was again markedly low in those birds fed a mixture of cholesterol and soy sterols. Plasma from the birds fed Diet 2 was hyperlipemic in appearance. Plasma from birds fed Diet 3 presented a normal clear appearance, in general, and only in a few instances was a slight lipemia noticeable. The livers of chicks fed Diet 2 showed, on a dry weight basis, an average cholesterol content almost 10 times that of the controls whereas those birds fed Diet 3 had liver cholesterol values less than twice as great as the controls. Livers from birds fed Diet 2 were extremely fatty in appearance, whereas Diet 3 livers were normal (non-fatty) in appearance.

The mechanism whereby these results are obtained is not apparent. The data would appear to suggest, however, that one or more of the constituents of the mixed soybean sterols interferes with absorption of cholesterol. The results obtained with the sitosterol fraction are identical with those obtained with the mixed soy sterols (Table I). Sperry and Bergmann(4) have noted that total cholesterol levels were lower in the livers of mice fed sitosterol than in control mice and suggested that perhaps sitosterol might interfere with the reabsorption of cholesterol secreted into the intestinal tract. It should be noted that normal growth was obtained on all diets used in these experiments.

Analytical data were obtained on a representative sample of mixed soy sterols by the Schoenheimer-Sperry procedure, using a sample of pure cholesterol[‡] as a standard of reference. The color intensity of the soy sterols was found to be, on the average, 77.3% of that of an equal weight of cholesterol. When saponification and the digitonin precipitation was used an average of 89% recovery was obtained with the mixed soy sterols. Thus, if any appreciable absorption of the mixed soy sterols occurred it would probably have been detected by the analytical method used. The data shown above (Fig. 1) indicate that there could be very little absorption of the constituents of the soy sterols in their original state.

The data presented in this report do not exclude the possibility that the effects described may be produced by some minor constituent of the sterol mixtures fed.

Summary. Evidence is presented that the addition to the chicks' diet of mixed soybean sterols and of purified sitosterols in the presence of cottonseed oil and cholesterol causes markedly lower plasma and liver levels of cholesterol than the addition to the diet of a mixture of cholesterol and cottonseed oil alone. The possibility is discussed that one or more of the constituents of the mixed soybean sterols interferes with the absorption of cholesterol.

The author wishes to acknowledge his indebtedness to Dr. Ruth Okey of the University of California for her interest and suggestions.

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[‡] Difco Standardized Cholesterol m.p. 149.5-150°C (corr.)

Quantitative Estimation of Antispasmodic Potency Using Isolated Guinea Pig Gall Bladder—Common Duct Preparation.[*][†] (19003)

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Reuse(1) evaluated the antihistaminic activity of pyrilamine and Phenergan with the isolated guinea pig gall bladder-common duct preparation. The data presented indicated that the log-dose response was linear but the method was not developed as a quantitative procedure for estimating antispasmodic potency.

In view of the fact that the antihistaminic drugs have varying degrees of antispasmodic activity(2), we have investigated their effects upon acetylcholine spasm of the guinea pig gall bladder-common duct *in vitro*. Statistical evaluation of the results indicates that this tissue preparation is useful for screening drugs for antispasmodic activity.

Experimental. Guinea pigs, of either sex, weighing 343 to 958 g (average 607 g) were killed by a blow on the head. The gall bladder and common duct were removed, emptied of bile and arranged for recording contractions by the Magnus method. The bathing fluid

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[*] The authors wish to thank Parke, Davis and Co. for the Benadryl and Ambodryl; Merck and Co. for the Phenergan; Bristol Laboratories for the C-5581-H; Winthrop-Stearns for the Thenfadil; Burroughs-Wellcome and Abbott Laboratories for the Chlorocyclizine; Schering Corp. for the Chloro-Trimeton; and Medico Chemical Specialties Corp. for the Anthallan used in this investigation.

[†] This article is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

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TABLE I. Antispasmodic Activity of Anthallan.

		Anthallan activity			
		Counteraction		Prevention	
Spasmogen	Conc. in mM	ED$_{50}$ and range in mM	Slope and range	ED$_{50}$ and range in mM	Slope and range
Histamine	12	4.1 (2.1–8)	2.59 (.66–10.1)	5.2 (2.6–10)	2.59 (.66–10.1)
BaCl$_2$	80	60 (54–312)	2.19 (.61–7.8)	150 (62–360)	2.2 (.61–7.9)
Acetylcholine chloride	.04	39 (15–100)	4.1 (1.5–8.5)	33 (12–86)	4.1 (1.5–8.5)

was Tyrode's solution of the following composition: NaCl, 8.0 g; KCl, 0.2 g; MgCl$_2$, 0.1 g; CaCl$_2$, 0.2 g; Na$_2$HPO$_4$, 0.05 g; NaHCO$_3$, 1.0 g, and dextrose, 1.0 g per liter. Maximum sensitivity of the tissue was obtained by allowing it to remain in the bathing fluid for 1½ hours after removal from the animal. The bathing fluid was changed every half hour during this interval. The spasm was produced by adding 0.5 cc of the spasmogen to the bath (see Table I for concentrations used). The antihistaminic solutions were added to the bath in a constant volume of 0.5 cc in concentrations varying from 0.0002 to 40 millimoles per liter. The drugs were evaluated for their ability to counteract as well as prevent the spasm, using five preparations for each drug. The results obtained were statistically analyzed by the Litchfield-Wilcoxon(3) method.

Results. Table I shows the results obtained when Anthallan was tested against musculotropic and neurotropic spasmogens. From these data it is quite evident that the guinea pig gall bladder-common duct preparation is more sensitive to neurotropic stimulus than to a musculotropic one. It can be seen that Anthallan is approximately 6 to 10 times more potent as an antihistaminic than it is as an antispasmodic. Against any of the spasmogens it was observed that the log dose-response curve was linear within a 10-fold dilution range, *i.e.*, 4 to 40 mM.

Inasmuch as the antihistaminic potency of the various drugs had been well established (2), our other evaluations were confined to establishing their antispasmodic potency compared to atropine. The results obtained are given in Table II. It is quite evident that although the antihistaminic drugs can counteract or prevent acetylcholine spasm, their potency in this respect is of a very low order. Phenergan is the exception, being less potent than atropine in counteracting such spasms but being equal to atropine in preventing them. Although it would appear from the ED$_{50}$ values that Ambodryl, C-5581-H and Thenfadil have equal potency in counteracting acetylcholine spasm, calculation of their potency ratios compared to atropine shows that Ambodryl is more potent than the other compounds. Similarly such calculations indicate that Thenfadil is more potent than C-5581-H in preventing such spasms. The drugs are rated in the following order in counteracting or preventing acetylcholine spasm: Atropine, Phenergan, diphenhydramine, Ambodryl, Thenfadil, C-5581-H, chlorocyclizine, chloroprophenpyridamine and Anthallan. The antihistaminic drugs gave linear log dose-response curves within a 100-fold dilution range, *i.e.*, 0.04 to 4 mM.

Discussion. Loew(4) has shown that the antihistaminic drugs can counteract histamine, acetylcholine or barium spasm of the isolated guinea pig ileum. The dose of antihistamine required is dependent upon the particular spasmogen used, being lowest with histamine and highest with barium. Our data on Anthallan (Table I) confirm these findings and show that the isolated guinea pig gall bladder-common duct preparation gives results quantitatively equal to the ileum preparation now commonly employed for evaluating antihistaminic and antispasmodic potency. Furthermore, the lack of spontaneous rhythmic contractions by the gall bladder-common duct preparation eliminates one possible

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TABLE II. Antispasmodic Activity of Antihistaminics.

			Counteraction		Prevention	
Drug	Chemical name	Molecular wt base	ED_{50} and range in mM	Potency compared to atropine	ED_{50} and range	Potency compared to atropine
Atropine	Tropine ester of tropic acid	289.2	.007 (.001–.032)	100	.0042 (.0009–.019)	100
Phenergan	[N-dimethylamino isopropyl-thio-diphenylamine]	284.4	.046 (.008–.25)	31	.005 (.001–.018)	100
Diphenhydramine	β-dimethylamino-ethyl benzhydryl ether	255.4	.37 (.06–2.41)	21	.68 (.11–4.08)	6
Ambodryl	β-dimethylamino-ethyl-(4-bromobenzhydryl)-ether	334.3	.74 (.08–6.3)	13	1.25 (.14–11.25)	4
C-5581-H Bristol	2-benzylphenoxy-N, N-dimethylethanolamine]	255.4	.72 (.24–2.2)	2	.8 (.2–3.2)	.9
Thenfadil	[N′-(2-pyridyl)-N′-(3-thenyl)-N, N-dimethyl ethylenediamine]	259.4	.6 (.13–2.7)	2	3 (.43–21)	1.6
Chlorocyclizine	[N′-methyl-N′-(4-chlorobenzhydryl) piperazine]	300.8	.8 (.16–4.1)	1	1.41 (.25–7.8)	.6
Chloroprophenpyridamine	[1-(4-chlorophenyl)-1-(2-pyridyl)-3-dimethylamino propane]	274.8	4.3 (1.4–12.9)	.3	2.1 (.9–5.9)	.3
Anthallan	3-di(n-butyl)-aminomethyl 4,5,6-trihydroxyphenyl phthalamide	323.2	39 (15–100)	.07	33 (12–86)	.05

source of error common to ileum preparations. The response of the gall bladder-common duct preparation to the various spasmogens and the concentrations of these agents required is of the same order of magnitude as required to elicit a similar response from the ileum preparation. The linearity of the log dose-response curves and the dosage range is similar for both preparations.

Previous reports have shown that the antihistaminic drugs have only a slight antispasmodic activity as compared to atropine(2,4-8). Sharp(8) found that diphenhydramine had a greater antispasmodic activity than Ambodryl. The results herein presented confirm these findings.

Summary. It has been shown that the isolated guinea pig gall bladder-common duct preparation is suitable for the evaluation of antispasmodic and antihistaminic activity. The log dose-response curve is linear over a wide range. The drugs studied are rated in the following order in counteracting or preventing acetylcholine spasm: Atropine, Phenergan, diphenhydramine, Ambodryl, Thenfadil, C-5581-H, chlorocyclizine, chloroprophenpyridamine and Anthallan.

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Effect of 2,6-diaminopurine on Virus of Russian Spring Summer Encephalitis in Tissue Culture.* (19004)

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The destructive effect of Russian Spring Summer Encephalitis (RSSE) virus infection on several transplantable tumors in mice has been reported(1,2). The mice eventually succumb to systemic injury from the virus after their tumors have been destroyed. Hence one phase of our program includes a search for agents with viricidal activity, which might be employed therapeutically after the oncolytic action of the virus is complete.

Compounds structurally related to the components of nucleic acid have been shown to interfere with the growth of micro-organisms (3-9). Since an infecting virus and its host cells are presumed to be in competition for nucleic acid metabolites such as purines and pyrimidines(10), or the enzymes utilizing them, a rational approach to the treatment of virus infection is to employ potential antimetabolites of nucleic acid. Thompson *et al.* (11-12) reported that the growth of vaccinia virus *in vitro* is inhibited by 2,6-diaminopurine (DAP).† That compound accordingly was employed in our study. The results indicate that DAP inhibits the multiplication of RSSE in tissue culture. Since no agent therapeutically active against this virus has been hitherto reported, the results of the study are presented herewith.

Methods. RSSE virus multiplies readily in a Maitland type tissue culture. This technic lends itself well to the assay of the effect of a compound on the proliferation of the virus as well as of the tissue. The cultures were set up in 50 cc Erlenmeyer flasks containing 0.5 cc of 10% horse serum, 4.5 cc Tyrode's solution, and about 100 mg of minced tissue. Two types of mouse tumors were used; the Crocker Sarcoma 180 and the Carcinoma 1025. Both minced mouse and chick normal embryonic tissues were employed. The tumor tissue was cut into 2 mm cubes and 10 pieces added to each flask. Tumor fragments from flasks inoculated with virus were implanted into mice immunized against RSSE virus during the course of the experiment as a test of tissue viability (bioassay). Unless otherwise stated, 0.1 cc of a 10% suspension of infected mouse brain was the inoculum. It was added at approximately the same time as the other tissue culture components, including the test compound. Penicillin and streptomycin were always added to prevent bacterial contamination. The contents of each flask were thoroughly mixed and incubated at 37°C. Control flasks containing no virus were run concurrently with each experiment. At 24, 48, and 72 hour intervals, designated flasks were removed from the incubator and the super-

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* This work was supported by funds from the National Cancer Institute of the U. S. Public Health Service, American Cancer Society and the Damon Runyon Memorial Fund for Cancer Research.

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† Obtained through the courtesy of Burroughs-Wellcome Laboratories.

TABLE I. Effect of Different Concentrations of DAP on Multiplication of RSSE Virus in Tumor Tissue Culture.

Hr in tissue culture	No DAP	Molar concentrations of 2,6-diaminopurine 10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-7}
24	5.5*	3.5	3.5	3	5.5	7
48	6.5	1.5	1	3	5.5	5.5
72	3	NV†	NV	.5	4	3.5

* Titers expressed as reciprocal of LD_{50}.
† NV designates no virus.

TABLE II. Effect of Adding DAP 24 Hr After the RSSE Inoculum.

	Hr after virus inoculation 24 hr	48 hr	72 hr
S-180 + virus	6.5*	7	5.5
+ DAP + virus	—	3	.5

* Titers expressed as the reciprocal of LD_{50}.

natant fluids tested for virus content by intracerebral inoculation of 0.03 cc into 3-4 week old mice. Titers were calculated in terms of the 50% endpoint by the method of Reed and Muench(13).

Results. Each of the experiments reported is representative of 3 or more studies.

Effect of different concentrations of DAP. Initially, different concentrations of DAP were added to the culture flasks containing RSSE virus with Sarcoma 180 as the tissue source to determine whether the agent was viricidal. It was found that a 10^{-3} M concentration inhibited the multiplication of virus only. At a 10^{-2} M concentration, both tumor growth and virus multiplication were stopped, whereas at a concentration of 10^{-5} M neither was affected. As a result of the data summarized in Table I, a concentration of 10^{-3} M of DAP was chosen for more detailed study of virus multiplication *in vitro*.

Effect of adding DAP after virus. When DAP is added to the tissue culture 24 hours after the virus inoculation, at the time when the tissue is infected and the virus is actively multiplying, the inhibitory effect is marked. As shown in Table II, the virus titered $10^{-6.5}$ when the DAP was added. Twenty-four hours later the titer had fallen, indicating that viral multiplication had halted. Only a small amount of virus could still be detected after 48 hours.

Recovery of virus from DAP-treated tissue cultures. Attempts were made to recover the virus after it had been in contact with DAP. When fresh tumor tissue was added to the treated cultures daily, or when the washed, previously infected tissue was placed in fresh Tyrode's solution with horse serum, no virus could be detected by intracerebral inoculation in mice. This was the case even though from 66% to 100% of the tissue was viable on bioassay and virus was present up to 48 hours. Clearly, the effect of DAP on the virus was irreversible under the circumstances employed.

Effect of adenine and guanine on the viricidal action of DAP. Inhibition of the growth of *L. casei* by DAP has been reported(6). The inhibition could be reversed by either adenine or guanine at low concentration of DAP. The effect was reversed by adenine but not by guanine at higher concentrations. The inhibition of vaccinia virus *in vitro* by DAP is prevented by adenine in equimolar concentration, but guanine is ineffective in this system(12). A similar effect has been observed in the *in vitro* inhibition of RSSE multiplication by DAP. When added to the tissue cultures simultaneously with DAP and RSSE, adenine, in equimolar concentrations, prevented the inhibitory action of the compound, but guanine failed to do so. The results of a typical experiment are presented in Table III.

Twenty-four hours after the virus had been exposed to DAP, equimolar concentrations of adenine and of guanine were added to each of 2 sets of culture flasks. As shown in Table IV, within 48 hours after the addition of adenine, the virus began to recover its ability to multiply. Guanine, however, proved to be ineffective in reversing the inhibitory effect.

Effect of 8-azaguanine. Since DAP was active in inhibiting the multiplication of RSSE, the effects of some structurally related compounds were ascertained. The results

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13. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

TABLE III. Effect of Adenine and Guanine on the Inhibitory Action of DAP in Tumor Tissue Culture.

	Hr in tissue culture					
	24 hr		48 hr		72 hr	
	% growth	Titer*	% growth	Titer	% growth	Titer
S-180	100	—	100	—	100	—
+ virus	66	5	30	4.5	10	3.5
+ DAP	100	—	83	—	0	—
+ virus	100	3.5	66	1	12	NV†
+ Aden.	100	—	83	—	0	—
+ Aden. + virus	83	5	66	5.5	83	3
+ Guan.	100	—	66	—	33	—
+ Guan. + virus	66	3.5	100	1.5	0	10^{-0}‡

* Expressed as the reciprocal of LD_{50}.
† NV indicates no virus detected.
‡ 1 of 3 mice inoculated with undiluted supernatant died of encephalitis.

TABLE IV. Partial Reversal of the Action of DAP on Multiplication of RSSE in Tumor Tissue Culture by Adenine.

	Titers*		
	24 hr	48 hr	72 hr
S-180 + virus	6.5	7	5.5
+ DAP + virus	2	NV‡	NV
+ virus + aden.†	—	1.5	3.5
+ guan.†	—	.5	.75

* Expressed as the reciprocal of LD_{50}.
† Adenine and guanine added 24 hr after 2,6-diaminopurine.
‡ NV indicates no virus detected.

obtained with 8-azaguanine‡ are typical and indicate that the compounds have no power to inhibit the multiplication of the virus. At the concentration used (10^{-3} M), 8-azaguanine exerted little effect on the growth of the sarcoma.

Tissue other than Sarcoma 180. In all the preceding experiments, Sarcoma 180 was the source of tissue. However, tumor from the culture flasks, both control and experimental, did not always give 100% growth on bioassay. In this system, one could not be certain that DAP itself had no effect on the viability of the tumor tissue. It was therefore necessary to employ a tissue that would be less sensitive to DAP than is the Sarcoma 180. It has been found that Carcinoma 1025(14) in tissue culture is relatively resistant to the action of DAP. Accordingly the studies were repeated with this tumor. Minced mouse and chick embryonic tissues were also used. The results are presented in Table V, including those obtained employing DAP and RSSE in a tissue-free medium. It is evident that the DAP had no effect on the virus *per se*, but does interfere with its ability to propagate in any form of tissue culture employed.

Discussion. The activity of 2,6-diaminopurine in the inhibition of Russian Spring Summer Encephalitis virus in cultures of normal and neoplastic tissues has been established. The mechanism of the action is still not clear. It is only when virus multiplies in the tissue that inhibition takes place. In the rat DAP has been shown to be a precursor of nucleic acid guanine(15), and to be highly toxic. Hence the viral inhibitory effect may be related to a block at some stage of nucleic

TABLE V. Effect of DAP on Multiplication of RSSE in Different Tissues.

	Titers*		
	24 hr	48 hr	72 hr
S-180 + virus	5.5	6.5	3
+ DAP	3.5	1.5	NV†
1025 + virus	7	6	6
+ DAP	3	3.5	4.5
Mouse embryo + virus	5.5	7	7
+ DAP	5.5	5	5
Chick embryo + virus	7	6.5	5.5
+ DAP	5	>6	3.5
No tissue + virus	2	.5	NV
+ DAP	2	.5	NV

* Expressed as the reciprocal of LD_{50}.
† NV indicates no virus detected.

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‡ Obtained through the courtesy of the American Cyanamid Co.

14. Berger, R. E., personal communication.

15. Bendich, A., and Brown, G. B., *J. Biol. Chem.*, 1948, v176, 1471.

acid metabolism. Since the virus in a tissue-free medium is not affected by DAP, the action is not a direct one. The data presented suggest that DAP interferes with some metabolic process in tissue related to nucleic acid synthesis, and one that is necessary for virus multiplication.

Biesele(16) has reported that DAP, in the concentration used in these studies, preferentially injures Sarcoma 180, as compared to mouse embryonic skin, in a roller tube tissue culture. Hence, it is necessary to exclude the possibility that the results presented here were due to death of the tumor cells caused by DAP and consequent removal of any medium capable of permitting viral multiplication. Four facts provide evidence against this possibility. (1) Although on bioassay most of the tumor is killed in the virus-infected control flasks without DAP, nevertheless the virus can be recovered in high titer. Hence very slight tissue growth permits viral multiplication. Extensive tissue growth in the DAP-treated flasks yielded no virus. (2) Such virus as may survive 48 hours of contact with DAP has lost its capacity to multiply when put into fresh media containing actively growing tissue. (3) Although the Carcinoma 1025 is relatively resistant to the action of DAP, inhibition of viral multiplication also occurs in this tissue. (4) DAP inhibits RSSE *in vivo*(17).

Summary and conclusions. (1) The multiplication of the virus of Russian Spring Summer Encephalitis in tissue culture is inhibited by 2,6-diaminopurine. (2) The action can be blocked as well as reversed by the addition of equimolar concentration of adenine. Guanine is not effective.

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16. Biesele, J. J., Berger, R. E., Wilson, A. T., Hitchings, G. H., and Elion, G. B., *Cancer*, 1951, v4, 186.

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Effect of 2,6-diaminopurine on the Course of Russian Spring-Summer Encephalitis Infection in the Mouse.* (19005)

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The compound 2,6-diaminopurine (DAP) has been shown(1) to be concerned in the synthesis of polynucleotide guanine. It also has been proved to have certain types of biological activity. It prolongs survival time of mice with transplantable leukemia(2). It is seriously injurious to the cells of Sarcoma 180 in tissue culture(3) in concentrations which have no effect on normal embryonic cells growing at the same rate although it exerts no demonstrable effect on the same tumor *in vivo*(4). It has also been shown to have an injurious action on the hematopoietic system of dogs(5) and swine(6) and chick embryo(7). It is known to inhibit the multiplication of vaccinia virus *in vitro*(8) while having no effect *in vivo*. The demonstration that it inhibits multiplication of Russian Spring Summer Encephalitis virus (RSSE) in

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TABLE I. Effect of Daily DAP Injection on Survival of Mice Infected with RSSE (Pre-virus Treatment).

	Dilution of virus, .05 cc i.p.	No. of animals	Living	Dead	% survival
Control	10^{-3}	20	0	20	0
DAP	10^{-3}	20	0	20	0
Control	10^{-5}	49	4	45	8.2
DAP	10^{-5}	48	15	33	31.2
Control	10^{-6}	65	12	53	18.5
DAP	10^{-6}	63	26	37	41.5
Control	10^{-7}	75	20	55	26.8
DAP	10^{-7}	69	42	27	61

TABLE II. Effect of Daily DAP Injection on Survival of Mice Infected with RSSE (Post-virus Treatment).

	Dilution of virus, .05 cc i.p.	No. of animals	Living	Dead	% survival
Control	10^{-5}	29	2	27	6.9
DAP	10^{-5}	29	9	20	31
Control	10^{-6}	141	26	115	18.4
DAP	10^{-6}	137	58	89	42.5
Control	10^{-7}	45	7	38	15.5
DAP	10^{-7}	46	19	27	41.5

culture of tumor tissue has led to the studies on intact animals here reported.

Materials and methods. Preparation of 2,6-diaminopurine.[†] The compound was made up daily by dissolving a concentrated suspension in appropriate amounts of saline. The usual amount administered was 70 mg/kg or 1.4 mg/mouse, a dose which had been found to be non-toxic when given daily for 10 successive days. The toxicity of each new preparation of DAP was tested. *Mice.* Animals weighing 18-22 g obtained from commercial breeders were used. No weight loss due to the chemical or to the virus was noted. *Virus.* Source of the RSSE was infected mouse brains maintained in the frozen state until used. A 10% suspension of brain was made in 10% normal horse serum saline and appropriate dilutions were inoculated into both DAP-treated and control animals.

Experimental. Pre-virus treatment. Mice were given 70 mg/kg of DAP in saline by intraperitoneal injection one or 2 days previous to the administration of virus. Groups were then infected by the intraperitoneal inoculation of 0.05 cc of RSSE diluted to 10^{-5}, 10^{-6}, 10^{-7}. Daily injection of DAP was continued until signs of the infection appeared; in most experiments for 10 days. Control animals received comparable injections of saline after infection with the same amount of virus.

A summary of 6 experiments is given in Table I. It is apparent from the results that there were more survivals among the animals which were treated with the DAP than among the controls. The difference is apparent in all 3 groups of animals, those receiving 10^{-5}, 10^{-6} or 10^{-7} dilutions of virus, and is statistically significant.[‡] When virus was inoculated at a 10^{-3} dilution, no difference between survival rates of treated animals and untreated controls was noted.

Post-virus treatment. Mice were inoculated with varying dilutions of virus intraperitoneally and treatment was begun 24 hours later by the injection of DAP according to the routine previously described. A summary of 5 experiments is presented in Table II and shows that the DAP, given after virus inoculation, still exerted a significant effect on the survival rates of the infected animals. When treatment was delayed until 48 hours after the inoculation of the virus, no benefit

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[†] This compound was supplied by the Burroughs-Wellcome Laboratories.

[‡] The statistical analyses were carried out by Miss Mary MacDonald of the Memorial Hospital Statistical Department.

TABLE III. Distribution of Virus in Animals* treated with DAP Compared with Untreated.

Time after DAP	Blood						Brain					
	Treated			Untreated			Treated			Untreated		
	Pos.	Prob.	Neg.	Pos.	Prob.	Neg.	Pos.	Prob.	Neg.	Pos.	Prob.	Neg.
2 hr	0	1	2	0	0	3	0	0	3	0	0	3
1 day	0	0	3	*2* [†]	0	2	0	0	3	0	0	3
2	*2* *1.5*	0	1	*3.5*	0	2	0	0	3	0	0	3
3	*2.5* *3.5*	0	1	*2* *2*	0	1	0	0	3	0	0	3
4	*2* *2*	0	1	*2.5* *2.5*	0	1	0	0	3	*1.5*	0	2
6	*2*	1	1	0	0	3	0	0	3	*5.5*	1	1
7	0	0	3	0	1	2	*3.5*	0	2	*6.5* *2*	0	1
	7	2	12	6	1	14	1	0	20	4	1	16

* Animals killed 2 hr after DAP administration.

[†] Italicized numbers indicate the amount of virus present as determined by intracerebral titration and expressed by the reciprocal of the LD_{50}.

was obtained.

Effect of varying dosage of DAP. When more than 70 mg/kg was given, the compound was not tolerated. When given at 60 mg/kg and if 0.05 cc of a 10^{-6} dilution of the virus was inoculated intraperitoneally, 30% of the treated animals survived, in contrast to 10% of the controls. At lower levels of DAP than this, no therapeutic effect was exerted. When the same amount of DAP was given in divided doses, no additive effect was obtained.

Effect of other routes of administration of DAP. When the DAP was given in daily subcutaneous doses before the intraperitoneally inoculated virus, a slight increase in the survival rate of treated animals over the controls was noted. The figure is not statistically significant. Inoculation of the DAP intracerebrally, followed by inoculation of the virus intraperitoneally 24 hours later, had no effect. The mechanical trauma of repeated intracerebral inoculation was not withstood by the animals. Hence it was impossible to determine if this route of DAP administration was effective. The same difficulty was encountered with intravenous inoculation.

Effect of varying routes of inoculation of the RSSE. The DAP was injected intraperitoneally at 70 mg/kg either before or after virus inoculation. When the virus was given subcutaneously, the same protective effect of the DAP was exerted as when it was inoculated intraperitoneally. When the virus was given intracerebrally the compound was ineffective.

Effect of varying the duration of treatment with DAP. The DAP was administered intraperitoneally at 70 mg/kg for varying periods. Treatment was begun 24 hours after the virus was inoculated. When the DAP was given for only 3 consecutive days, no effect could be observed. When a dose was given every other day for 8 days, the results were as good as if the DAP had been given daily for 7 days. When the compound was administered before or 24 hours after the virus, the treated animals survived approximately one day longer than the control animals. Therefore there appeared to be no significant difference in survival time between the animals treated with DAP before, and those treated after, administration of the virus.

Effect of DAP on other neurotropic virus infections. The DAP had no therapeutic effect against 3 other viruses which infect mice when inoculated intraperitoneally. These are West Nile Encephalitis, Ilheus Encephalitis and Louping Ill. The ineffectiveness of the DAP against the Louping Ill virus was surprising since it is so similar to RSSE that some consider them to be identical. The strain of Louping Ill used in our laboratory differs from the RSSE. The Louping Ill virus is much less infectious when given by the intraperitoneal route than is our RSSE. The DAP was also

TABLE IV. Distribution of Virus in Animals[*] Treated with DAP Compared with Untreated.

Days after DAP	Blood				Brain			
	Treated		Untreated		Treated		Untreated	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
1	*.5*[†]	2	*1.5* *1.5* *.4*	0	0	3	0	3
2	*.25* *1.25*	1	>*2* *1.5* *1*	0	0	3	0	3
3	*1* *.7*	1	*3.5* *1.5*	1	0	3	*1.33*	2
4	*1.25* [illegible] *1.5*	0	*3.5* [illegible] *2.5*	0	0	3	*2.4* *4*	1
5	*1* [illegible]	1	*1* [illegible]	1	*4*	2	*3.5* *4*	1
6	*.5*	2	*1.59* *1.5*	1	*7* *4*	1	*3.5* *5.5* *6*	0
7	0	3	*1.5*	2	*8* [‡]	2	*5.5* *8* [‡]	1
8	0	4	0	4	*8* [‡] *8.5*[‡]	2	*6* [‡] *8* [‡] *7* *7*	0
9	0	3	0	4	*4* [‡] *8* [‡]	2	*8* [‡] *8* [‡]	2
10	*.44*	2	0	4	>*9* [‡] >*9* [‡]	2	>*9* [‡] >*9* [‡]	2
Total	12	19	16	17	10	23	18	15
Toxic	2							

[*] Animals killed before administration of DAP.

[†] Italicized numbers indicate amount of virus present as determined by intracerebral titration and expressed as reciprocal of the LD_{50}.

[‡] Paralyzed or sick when killed.

ineffective against infection with Lansing poliomyelitis virus and Bunyamwera virus, inoculated intracerebrally.

Distribution of RSSE in treated and untreated animals. In an effort to find out whether there was any different distribution of RSSE in a group of mice treated with DAP from that of an untreated group, 100 mice were inoculated with 0.05 cc of a 10^{-6} dilution of virus intraperitoneally. The following day they were divided into groups of 50 each. One was given daily intraperitoneal injections of DAP (70 mg/kg) and the other of saline. Each successive day 3 mice from each group were killed 2 hours after they had been injected with the DAP. Their brains and bloods were inoculated intracerebrally in normal mice as a test for the presence of RSSE. If any indication of virus was found, the tissues were retested and their virus content established.

The results are recorded as follows: No virus: less than 50% deaths at 10^{-1}. Probable virus: more than 50% deaths at 10^{-1} dilution; and definite virus: virus present after dilution. The results are given in Table III. They show that there was little difference between the two groups in the concentrations of virus in the blood. The absence of virus in the brains of the treated group appears to be significant. The experiment was repeated in the same way except that a group of animals were sacrificed daily on successive days 2 hours before rather than after the injection of the DAP. When the animals became ill, 4 were killed from each group, 2 paralyzed, and 2 apparently healthy. When virus was present, its concentration was titrated (Table IV). In general, the same type of results was obtained. A comparison of the number of animals in the treated and untreated groups showing virus in the blood demonstrated the following: 19 of 31 (61%) were negative in the treated group (2 were toxic and could not be tested). In the untreated group, 17 of 33 (51%) were negative. Although in the treated group both the number of mice having virus in the blood, and the amounts of virus as determined by titration, were slightly less than in the untreated controls, the differences were not nearly so marked as those found in the brains. Of the treated group, the brains of 23 of 33 animals, or approximately 70%, were negative in contrast to the untreated, in which 33 of 55, or 45.5%, were negative. However, animals of both groups, when paralyzed, showed no significant difference in the amount of virus present in the brains.

Discussion. The effects of treatment with 2,6-diaminopurine of mice infected with Russian Spring-Summer Encephalitis virus have been investigated. The chemical, a substance which is involved in nucleic acid synthesis, has been shown(9) to have an effect on the proliferation of the virus in tissue culture. It has now been demonstrated that DAP exerts a therapeutic effect on animals infected with RSSE even if treatment is begun 24 hours after the virus has been inoculated.

The mechanism of action of the DAP is unknown, but the studies on the distribution of the virus in brain and blood of the treated and untreated animal gives us a clue as to the point of action of the substance.

Since as much virus is present in the blood of the treated animals as the untreated, DAP apparently does not have an effect on the invasiveness of the virus. When the virus is inoculated intracerebrally, the DAP is ineffective, and treated animals which are not protected by the DAP have as much virus in their brains as do the untreated controls. Hence the DAP exerts its activity either on the "blood-brain" barrier or directly on the cells of the brain.

Summary. The compound 2,6-diaminopurine when administered in doses of 70 mg/kg caused an increase in the survival rates of mice infected with Russian Spring-Summer Encephalitis by the intraperitoneal route. It was effective when treatment was started 24 hours after the inoculation of the virus.

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Metabolism of Amines. II: Mitochondrial Localization of Monoamine Oxidase. (19006)

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Despite numerous investigations of its enzymatic function, monoamine oxidase has not been significantly purified. As with other systems that are bound to insoluble particles, the difficulty lies in the extraction of sufficient amounts of active protein for the application of classical methods of fractionation. On the other hand, this difficulty implies a degree of association with organized structures that deserves investigation and suggests that the chemical neighborhood of the enzyme should be explored with fractionations that preserve the integrity of cytological units.

Previous investigators, notably Claude, Hogeboom, Schneider, Dounce, and their collaborators, have recognized the power of morphological fractionation. (See Schneider and Hogeboom(1) for review). In the present investigation it remained to apply their methods and correlate with their results.

Procedure. Adult male rats (170-230 g) of a Sherman strain were used in all experiments. After a week of observation with an unrestricted diet of Purina fox chow, each animal was allowed water during a final period

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of 18 hours and then was sacrificed by decapitation. The liver was removed immediately and taken into a cold room (0.-3°C) for centrifugal fractionation in 0.88 M sucrose, according to the method of Hogeboom *et al.*(2). As a preliminary step, the liver was passed through an Altmann press to remove connective tissue. About 3.0 g of the pulp were weighed quickly into a Potter homogenizer tube(3), sucrose solution was added to a final volume of 30.0 ml, the preparation was homogenized for about 5 minutes with a motor driven pestle, and then centrifuged. At each stage of the fractionation process, indicated in Fig. 1, the supernate was separated from the precipitate by decantation, fresh sucrose was added to the precipitate and the latter again was dispersed by a motor driven lucite paddle. Since the amount of sedimentation effected is determined by the product of centrifugal force and time, within wide limits of variation of either factor, it was convenient to plan the work in terms of "centrifugal units" (relative centrifugal force × time in minutes × 10^3)(4). In terms of these units the components of the homogenate were separated into three classes: (1) those that were sedimented consistently by 6 units (fraction N), (2) those that remained suspended after 6 but sedimented with 400 units (fraction M, and the fraction NW which had been swept down once with the larger particles but on redispersion was indistinguishable from M), (3) the supernate (S) consisting of fine particles and soluble material, which remained suspended during repeated centrifugations of 400 units.

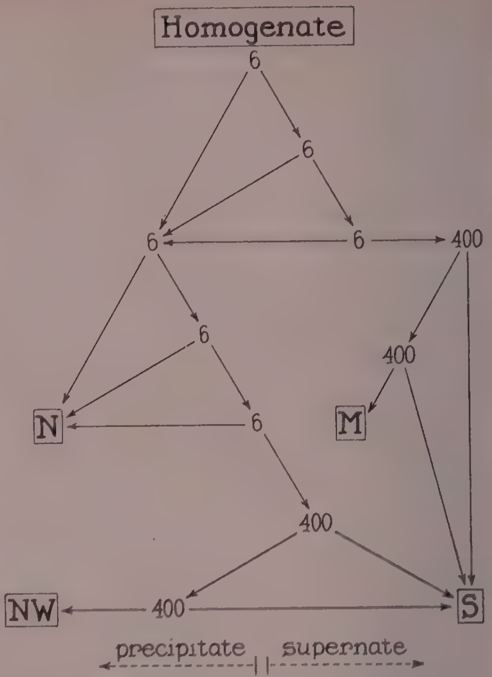

FIG. 1. Diagram of the centrifugal fractionations in 0.88 M sucrose. The numbers at each junction indicate the centrifugal units (1000 G × min) (4) that were applied to a suspension; the emergent arrows trace the fate of the separated portions, sediment to the left and supernate to the right. Fractions that were analyzed are enclosed in squares.

The activity of monoamine oxidase in the original homogenate, and in each of four final fractions (N, M, NW, S), was determined by the rate of ammonia release from tyramine(5). Nitrogen was determined in the earlier experiments by the microdiffusion method of Conway(6) and later by direct nesslerization, as applied by Eder(7) to the mercury-catalyzed digestion mixture of Hiller *et al.*(8). An experiment was considered satisfactory if the enzymatic activity and the nitrogen content of the original homogenate were accurately recovered in the summation of values of derived fractions (Table I). Because of the invariable occurrence of intact cells and cytoplasmic fragments in the nuclear fraction (N), it was not possible with this method to determine whether or not enzymatic activity was contained in the nucleus

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TABLE I. Distribution of Monoamine Oxidase.

Fraction	Enzyme Units / g wet tissue	 % of homogenate	Nitrogen mM N / g wet tissue	 % of homogenate	Specific activity Units enzyme / mM N	 % of homogenate
N	5.3 ± 1.6	21.9	.49 ± .18	23.1	11.7 ± 3.1	98 ± 17
NW	3.1 ± 1.5	12.9	.11 ± .06	5.2	33 ± 18	312 ± 218
M	13.7 ± 1.8	56.8	.56 ± .15	26.4	26 ± 6.9	219 ± 25
S	1.9 ± 1.2	7.9	.91 ± .33	42.9	2.6 ± 2	20 ± 14
Sum	23.8	99.5	2.07	97.6		
Homogen.	24.1 ± 2		2.12 ± .38		11.9 ± 2.6	

The fractions are those obtained by differential centrifugation in 0.88M sucrose(2). The numbers are the means and stand. dev. from 13 exp.

in addition to its predominant localization in the adherent mitochondria. To test this point attempts were made to purify the nuclei contained in this fraction. Rehomogenization at different acidities and differential centrifugation in 0.88 M sucrose yielded preparations that were inferior to those obtained by the citric acid method of Dounce(9); the latter, therefore, was employed for the study of nuclei free from detectable cytoplasm. Every analyzed fraction was examined microscopically, both in the fresh state with a phase contrast microscope and after fixation with 4% osmic acid and staining with hematoxylin, Giemsa or Wright's stain.

Observations. It may be seen in Table I, which summarizes 13 concordant experiments, that the enzymatic activity became concentrated in the mitochondrial fractions, M and NW. The specific activity (units of enzymatic activity per unit of nitrogen) in fraction M averaged 219% of the specific activity of the original homogenate. The nuclear washing fraction NW was histologically identical to fraction N; when the activities contained in these two fractions were summed it was found that from 66 to 75% of the total activity had been recovered with isolated mitochondria.

The remainder of the original activity passed into the fraction N, which consisted of nuclei with variable appendages of cytoplasm, unbroken cells and small amounts of connective tissue fragments and red blood cells. Separate experiments, in which negligible specific activities were found with blood, subcutaneous connective tissue and choroid plexus, made it unlikely that connective tissue or vascular elements in the fraction contributed to any important extent. Similarly, it was found in 4 experiments that purified nuclei, prepared by the citric acid method, lacked appreciable enzymatic activity.

Upon histological examination of the different fractions, it was found that those prepared in 0.88 M sucrose retained the normal structure and staining properties of the formed elements, including the nuclei of fraction N. The cytoplasm-free nuclei, obtained after exposure to citric acid, were markedly altered, however; phase microscopy revealed in them opaque strands which were lacking in fresh liver nuclei, and in the fixed material it was seen that they had suffered a considerable loss in their capacity to stain with hematoxylin and with methylene blue.

Discussion. The data indicate that the enzymatic activity is predominantly associated with the mitochondria, and suggest that it might be confined to these elements. If it be assumed that the activity which passed into fraction N was carried by the numerous mitochondria in the cytoplasmic fragments it is possible to explain why the specific activity of this fraction remained the same as that of the original homogenate: both mitochondria and the inactive, protein-rich supernate (fraction S) had been removed in equivalent amounts. On the other hand, if the nuclei had a moderate or high specific activity in addition to that contributed by the mitochondria, it might be expected that the resultant specific activity of the fraction would have been greater than that of the original homogenate. The absence

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of significant enzymatic activity from the cytoplasm-free nuclei also favors the interpretation that the locus is exclusively mitochondrial, but it must be conceded that these nuclei could have suffered extraction of enzyme in the course of purification.

It is of interest to note that the mitochondrial localization of monoamine oxidase might have been expected from the precedent of other systems which transfer electrons to oxygen: d-amino acid oxidase(10), uricase (11), and processes dependent upon cytocrome oxidase(12). This convergence of oxidative systems suggests that the mitochondrion is a favorable location for the reduction of oxygen; whether or not there is a more intimate functional association between these processes and an enzyme that deaminates potent pharmacological agents is a question that awaits future investigation.

Summary. (1) The monoamine oxidase activity of rat liver cells is localized predominantly, and perhaps exclusively, in the mitochondria. (2) In this respect it conforms to a precedent since other aerobic processes have been shown to have a mitochondrial localization.

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Streptococcal Bacteriostatic Antibody in Patients Treated with Penicillin.*

(19007)

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The antibacterial activity of the serum of patients infected with the beta-hemolytic *Streptococcus* has been studied by a number of investigators. Bacteriotropins for this organism were first demonstrated by Hare(1). Kuttner and Lenert(2) noted that the bacteriostatic antibody was type-specific, persisted for many months, and was unrelated to the development of antistreptolysin O. From a detailed study of 3 patients, Rothbard(3) concluded that this immune substance first appeared in the third to fifth week following infection, was present for at least 37 weeks and was type specific. A higher incidence of bacteriostatic antibody in rheumatic fever patients than in those with uncomplicated streptococcal infections was observed by Rothbard, Watson, Swift, and Wilson(4). The close association between the presence of type-specific nucleoprotein (M) in the infecting *Streptococcus* and the later development of antibacterial immunity has been recognized(5-7).

The administration of penicillin to patients with acute streptococcal pharyngitis suppresses the formation of antistreptolysin O and anti-streptokinase(8-12). The work reported in this paper was carried out for the purpose of determining whether antibacterial immunity was affected in the same manner as the antibodies for the soluble products of the organism, by therapy with penicillin. The results indicate that depression of formation of bacteriostatic antibody may follow the exhibition of antibiotic and that the effect is related in degree to dose and route of administration.

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* This study was supported by a grant from the Schenley Laboratories, New York.

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Methods. The streptococci used in the bacteriostatic tests were obtained from 2 sources: (1) direct isolation from the respiratory tract of patients with scarlet fever, and (2) stock strains of known type. The latter were employed only when the initially isolated organism was lost; in these instances, the serological type of the original isolation had been determined and a stock culture of the same type was substituted. Most of the stock strains maintained the matt colonial form. Those isolated from patients tended to become smooth and were passed through mice periodically to reestablish the virulent type. Only matt-mouse virulent strains were studied. The bacterial suspensions used in the determination of antibacterial antibody were prepared in the following manner: An actively-growing 12-15-hour-old culture in yeast-tryptose-heart infusion broth was centrifuged and the sediment resuspended in the same medium. Dilutions of this suspension—10^{-2} to 10^{-6}, varying with the individual organism—were made in broth and fresh human blood added. The indirect bacteriostatic test was carried out according to the method of Todd(13) as modified by Ward(14) and Rothbard(3). The reaction was considered positive if a 2+ difference in growth was observed in at least 2 dilutions. The blood was obtained from individuals who did not have streptococcal disease and was not used unless examination revealed the absence of bacteriostatic antibody against the type of *Streptococcus* employed in the test. The direct determination of bacteriostatic antibody was made according to the procedure of Kuttner and Lenert(2). Controls consisted of bacterial suspensions without blood and plasma plus bacterial suspension. The serums studied were obtained from cases of scarlet fever at the time of admission to the hospital and at periodic intervals thereafter and were stored at —20°C until used. All of the patients were treated with penicillin. Blood was drawn, therefore, just prior to an injection—at least 12 hours after a dose—and examined for antibiotic activity before use in the test; detectable quantities of drug were never present. Only those persons from whom the beta-hemolytic *Streptococcus* was isolated at the time or within 24 hours of admission were studied. They were treated in the following manner: Twelve were given 250,000 units of the drug intramuscularly every 12 hours for 10 days; 19 received the same quantity of antibiotic at the same interval orally. In 12 cases 150,000 units was administered parenterally twice daily for 10 days; 11 were treated with a similar quantity by mouth for one week.

Results. All of the strains of *Streptococcus* studied belonged to group A and included, in order of frequency, types 3, 1, 4, 5, 12, 19, 23 and 30. Twenty-three per cent of the entire group of patients revealed demonstrable bacteriostatic antibody. In 3 instances this was detected only by the direct method; in 15 both the indirect and direct tests were used. In the remainder only the indirect procedure was employed.

Examination of Table I shows that the appearance of bacteriostatic antibody was related to the quantity and route of administration of penicillin. Of those who received 250,000 units of antibiotic intramuscularly, none developed type-specific antibacterial immunity. The administration of the same dose of drug by mouth, however, permitted the appearance of antibody in 21%. A moderate difference in the occurrence of antibacterial

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TABLE I. Relation of Dose and Route of Administration of Penicillin to Development of Bacteriostatic Antibody.

Penicillin units, ×1000	Route of admin.—duration of treatment	No. of patients	% of patients developing bacteriostatic antibody
250	Intramuscular—every 12 hr for 10 days	12	0
250	Oral—every 12 hr for 10 days	19	21.1
150	Intramuscular—every 12 hr for 7 days	12	33.3
150	Oral—every 12 hr for 7 days	12	41.5

activity in the serum was also detected when 150,000 units of penicillin was administered by different routes. The influence of the quantity of drug used is illustrated by the fact that of all of the cases receiving 250,000 units, regardless of route of administration, only 12.9% developed bacteriostatic antibody while the serums of 37.5% of those given 150,000 units were bacteriostatic. In practically all instances in which an antibacterial effect of the serum was demonstrable, the level of activity was considerably less than that observed by Rothbard(3) in untreated patients. In only one case in the present study was the titer high; this was a youngster with scarlet fever due to a type 12 *Strep. pyogenes* in whom the organisms persisted in the pharynx for 8 days.

Bacteriostatic antibody was first detected 3 weeks after the onset of infection in 6 cases; in 3 it appeared only after 9-15 weeks. In 5 patients antibacterial activity of the blood was demonstrable in the first or second week of illness and disappeared at the time of convalescence. This probably represents the nonspecific immune response reported by Tillet (15) in the acute phase of streptococcosis. In the remainder of the group bacteriostasis became evident initially between the 4th and 8th weeks. Bacteriostatic antibody persisted for at least 15 weeks in all of the patients; in 2 it was still present after 42 weeks. In 4 cases it was detectable after 15 but not after 24 weeks. These findings suggest that penicillin not only may inhibit completely the development of antibacterial immunity but may also decrease the quantity and shorten the duration if the antibody appears.

The serological varieties of beta-hemolytic *Streptococcus* present in those with positive bacteriostatic tests were type 3 in 4 cases, type 12 in 4, type 5 in 2, and types 1 and 3 in the remainder. Persistence or recurrence of streptococci in the pharynx did not influence the development of antibody, with the possible exception of the one patient cited above.

Serial determinations of antistreptolysin O and anti-hyaluronidase titers revealed no relation between their development and that of the antibacterial antibody since they were found just as frequently in the absence as in the presence of bacteriostatic activity in the serum.

Discussion. The results obtained in this study indicate that the administration of penicillin to patients with streptococcal infection suppressed the appearance of bacteriostatic activity. An indirect relationship between the quantity of drug used and the level of antibacterial immunity was apparent. When antibody was present, the titers were usually lower than those reported in patients receiving no specific therapy. The development of antibacterial activity was inhibited to a greater degree by the intramuscular than the oral route of administration of antibiotic. No relation between anti-hyaluronidase, antistreptolysin O and bacteriostatic antibody was noted. These results confirm the findings of Jawetz(16) in mice. The depression of antistreptococcal antibody production by penicillin is probably related to the rapid elimination of the infecting organisms(8). The higher incidence of antibacterial antibody in patients given smaller doses of drug or treated by mouth tends to support this possibility since both of these result in slower eradication of the bacteria and longer persistence of the antigenic stimulus than when large quantities or the parenteral route are used. The import-

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ance of the time of contact between the host and organism prior to treatment in the development of immunity has been pointed out by Harrison(17) and Curnen and MacLeod (18). Although the production of serum precipitins and mouse protective antibodies in pneumococcal infections in man is not affected by the exhibition of sulfonamide or penicillin, there is some depression of antibody formation if animals are treated early(19). With delay in therapy or the use of a very large infecting inoculum immunity develops to a satisfactory level(19).

Although only a very small number of patients developed secondary streptococcal complications, there was no correlation between the appearance of clinical or bacteriologic relapse and the development of bacteriostatic antibody. In one case only was there an indication that a suppurative sequela may have influenced the degree of antibacterial immunity.

The inhibition of the development of bacteriostatic antibody in patients ill with streptococcal infections by penicillin therapy probably is of greater clinical importance than a similar depression of anti-streptolysin O, anti-streptokinase and anti-hyaluronidase. The antibacterial antibody protects against reinvasion by a homologous serological type; the other antibodies probably play little or no role in the defense against invasion by the organism. Certain schedules of penicillin treatment, therefore, actually depress the development of effective immunity and permit reinfection by the same serologic type of *Streptococcus* which caused the initial disease.

Conclusions. (1) The administration of penicillin to patients with streptococcal pharyngitis depresses the formation of type-specific antibacterial antibody. (2) There is a relation between the dose and route of administration of penicillin and the degree to which antibacterial activity develops. (3) Suppression of antibody formation by penicillin is probably due to rapid removal of the antigenic focus. (4) Failure to develop type-specific antibacterial antibody for the *Streptococcus* following penicillin therapy increases the risk of subsequent infection by homologous serological types.

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Microwave Diathermy Treatment of Frostbite. (19008)

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Several investigators(1-5) have demonstrated the beneficial effect of rapid thawing with warm water on frostbitten tissue with respect to necrosis.

The purpose of this investigation was to determine the effect of immediate rewarming of frostbitten tissue with microwave diathermy on the incidence and extent of necrosis.

Materials and methods. The experiments were performed on 78 male albino rabbits of more than 2,500 g body weight, of which 39 were treated with diathermy and the remainder served as controls. Two degrees of cold

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TABLE I. Results with Diathermy Treatment of Local Cold Injuries.

No. of animals	Exposure temp (°C)	Treatment	Survival time	Muscle necrosis (%)	Skin necrosis (cm²)
12	–12°	Diathermy	4 wk	1.3 ± .5	0
[illegible]		Controls		5.[illegible] ± 1.8	0
25	–15°	Diathermy	8-9 days	45.7 ± 4.4	4.6 ± 1.5
24		Controls		61.9 ± 4	12.3 ± 2.1

exposure were used. —12°C for 30 minutes for the determination of muscle necrosis and —15°C for 30 minutes for the determination of muscle and skin necrosis. The exact procedure is given elsewhere(6). Muscle necrosis was determined in per cent and skin necrosis in square centimeters as described previously (7). After exposure to cold, the frozen legs were immediately treated with microwave diathermy* (wave length 12.2 cm in the 2,400 to 2,500 megacycle band) in the following manner: The corner reflector-type director was employed so that the 6-cm electrode was 1½ inches above the skin and parallel to the long axis of the leg which was laid flat on a table with the external surface up. At this distance 6 to 8 square inches receive 50% or more of the heat output, according to the manufacturer, which is more than the lateral surface area of the leg from ankle to knee. All exposures were for 20 minutes with a power setting of 40%. The tendency for the microwave energy to concentrate in the small areas of the body being treated resulted in burning of the ankle with the first trials. To avoid this mishap, the shape of the leg was transformed approximately to that of a cylinder by wrapping a single sheet of muscle from a rabbit's abdominal wall around the distal half of the leg and tying it in place. Except for rewarming all animals received similar care. The legs were covered with 3% sulfamylon ointment and sterile dressings applied which were changed daily. The animals exposed to —12°C were sacrificed after 4 weeks and those exposed to —15°C after 8 to 9 days.

The average results are given with the standard deviation of the mean.

For the statistical analysis, a nonparametric test of significance was used which gives a statistic which is distributed approximately as Student's "t"(8).

Results. Twenty-four rabbits had one hind leg exposed to an alcohol bath at —12°C for 30 minutes for the determination of the effect of diathermy on the extent of muscle necrosis. Twelve were treated with short waves as described above, and 12 were allowed to thaw at room temperature in air. Two control animals died before the extent of necrosis was apparent. Two control and 6 diathermy-treated animals escaped muscle necrosis but were included in the calculations. The results are given in Table I. The lesser muscle necrosis obtained in the diathermy-treated animals is statistically significant at the 1% level (P <.01 with 20 degrees of freedom).

For the determination of the extent of skin and muscle necrosis, another series of 54 rabbits was exposed to —15°C for 30 minutes. Twenty-seven were immediately rewarmed with diathermy, and 27 were thawed at room temperature in air. Two treated and 3 control animals died before the extent of necrosis was apparent. The extent of muscle and skin necrosis is given in the table. The difference in the extent of skin necrosis in the treated and untreated animals is significant (P = .01 with 47 degrees of freedom). Five control and 12 diathermy-treated animals did not develop skin necrosis, but were included in the computation.

The extent of muscle necrosis also showed

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a significant decrease in the diathermy-treated rabbits. ($P = .01$ with 47 degrees of freedom.) All animals in the treated and control groups showed muscle necrosis.

Discussion. Rapid rewarming of frostbitten rabbit legs with diathermy resulted in less extensive necrosis of skin and muscle in comparison to slow thawing in air at room temperature.

The manner in which rapid rewarming of frostbitten tissue is effective in reducing the incidence and extent of necrosis is controversial. We agree with Crismon and Fuhrman (9) that the reduction in the extent of necrosis obtained by rapid rewarming must be due to some action other than vasodilatation. These investigators pointed out that the results from rapid rewarming of frozen rabbit ears were superior to those obtained by stellate ganglion block. The poor results obtained by Finneran and Shumacker(4) by denervating frostbitten rabbit ears and their reported good results from rapid rewarming suggest the same conclusion. However, Lempke and Shumacker(3) hypothesized that the beneficial results in frozen mice tails obtained by rapid rewarming plus the vasodilatory effect of either tetra-ethyl-ammonium chloride[†] or sympathectomy are due to the prevention of tissue ischemia and subsequent thrombosis.

It is difficult to interpret these beneficial results attributed to vasodilatation in the light of the favorable results reported by Crismon and Fuhrman(10) from the local application of plaster casts or pressure dressings to frostbitten rabbit feet.

The shortening of the exposure time to cold by the rapid rewarming may be a factor, although Fuhrman and Crismon(2) doubt this explanation since rewarming in water at 36°C did not produce beneficial results in contrast to rewarming at 42°C.

That some metabolic tissue process must be favorably influenced by the local application of heat to the frozen tissue seems to be the best explanation for the beneficial results.

Conclusions. Rapid rewarming of frostbitten rabbit legs by means of short wave diathermy after two standard cold injuries resulted in a decrease in the extent of muscle and skin necrosis in comparison to control frost-bitten animals thawed at room temperature in air.

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Inhibiting Effect of Inositol on Serum Cholesterol and Phospho-Lipids Following Cholesterol Feeding in Rabbits.*† (19009)

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The role that inositol may play in cholesterol metabolism and in atherosclerosis has not been determined. Herrmann(1) found that inositol lowered the cholesterol in serum, aorta, heart and liver of old hens and(2) in the serum of hypercholesteremic patients. We(3) have found a similar effect in hypercholesteremic diabetic patients, as have Leinwand and Moore(4). On the other hand, Lupton *et al.*(5) found no effect of inositol on

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† This paper was presented in part at meeting of Amer. Chem. Soc., Chicago, Ill., 1950.

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serum cholesterol levels in diabetic patients; initial levels were not given. Stamler *et al.*(6) found that inositol did not prevent hypercholesteremia and hyperlipemia in cholesterol-fed chicks and(7), did not affect tissue or plasma lipid concentrations or severity of lesions in either spontaneous or stilbestrol induced atherosclerosis. Broun *et al.*(8) found a daily dose of 40 mg of inositol without effect on blood lipids in cholesterol fed rabbits. Davidson *et al.*(9) found inositol did not prevent hypercholesteremia or lesions in dogs fed cholesterol and thiouracil. In view of this controversy, it was decided to determine the effect of inositol on the rise in serum lipids known to occur in cholesterol fed rabbits.

Methods. Young female rabbits, about 4 months old, were maintained during the experimental period on a diet of Purina rabbit chow and hay. In the first experiment, the rabbits were divided into 2 groups. Group A (16 animals) received 1 g of cholesterol, while group B (7 animals) received 1 g of cholesterol plus 0.5 g of inositol daily except Sunday. Both cholesterol and inositol were administered in gelatin capsules which had been previously dipped in molasses to improve palatability as suggested by Pollak(10). After 132 days, some rabbits were sacrificed for pathological examination; the results will be reported later. In the second experiment, the remaining rabbits were given a different feeding regimen for 63 additional days; 11 rabbits from group A were fed 2 g of cholesterol plus 1.2 cc of salad oil, daily except Sunday, and 3 rabbits from group B were

TABLE I. Serum Lipid Values of Rabbits (on Regimens for 132 Days).

	Group*		Days							
			0	19	35	55	70	91	112	132
Total cholesterol, mg/100 cc	A	Avg	110	130	211	285	387	409	441	481
		Range	71–246	72–340	85–390	120–727	171-1282	223-855	260-871	292–940
	B	Avg	83	83	88	93	113	119	120	226
		Range	68–107	68–111	63–120	66–136	85–144	99–147	104–162	106–855
Cholesterol esters, mg/100 cc	A	Avg	80	95	154	203	279	290	312	334
		Range	52–180	52–267	62–299	86–541	116–1015	160–616	178–621	193–663
	B	Avg	59	59	62	64	78	81	82	154
		Range	48–72	49–69	44–74	47–88	61–125	68–113	68–108	70–577
Ratio, chol. est./ tot. chol.	A		.73							.69
	B		.72							.69
Lipid phosphorus, mg/100 cc	A	Avg	4.2	4.6	6.5	7.9	9.3	9.7	9.9	10.3
		Range	2.3–6.3	2.5–9.4	3.4–10.3	4.6–15.6	5.3–20.6	5.4–18.4	5.6–18.4	5.8–18.8
	B	Avg	4.1	4.2	4.4	4.5	4.7	4.7	4.7	5.2
		Range	3.1–5.4	3.1–5.5	3.4–5.6	3.3–6	3.5–6.1	3.8–6.2	3.9–6.2	3.9–8.8
Molar ratio, Tot. chol./lip. phos.	A		2.13							3.75
	B		1.70							2.14

* The 16 rabbits in Group A received cholesterol, the 7 in Group B cholesterol + inositol.

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TABLE II. Serum Lipid Values on Rabbits (on Regimens for 63 Days).

	Group*		Value at start	Days 21	42	63
Total cholesterol, mg/100 cc	A	Avg	424	564	929	1050
		Range	292–628	372–823	710–1340	748–1563
	B	Avg	190	198	338	370
		Range	110–323	119–333	202– 427	217– 500
Cholesterol esters, mg/100 cc	A	Avg	288	392	659	739
		Range	193–443	259–577	500– 941	518–1136
	B	Avg	134	138	235	252
		Range	75–233	78–241	134– 307	147– 336
Lipid phosphorus, mg/100 cc	A	Avg	8.7	9.9	14.7	16.3
		Range	5.8–11	6.6–15.5	10.8–18.1	12.2–25
	B	Avg	6.3	6.4	8.2	8.6
		Range	4–8.8	4.2– 8.8	6.6– 8.8	6.7– 9.5

* The 11 rabbits in Group A received cholesterol and salad oil, the 3 in Group B these components + inositol.

given these dietary supplements plus 1 g of inositol. An inanition period of 18 hours preceded all blood sampling. Blood specimens were obtained from the marginal ear vein after hyperemia was produced by application of a small amount of xylene. On the day prior to the initial feeding of cholesterol and inositol and at approximately three-week intervals thereafter, blood specimens were obtained for the determination on serum of total cholesterol and cholesterol esters by the method of Sperry and Brand(11), and lipid phosphorus by the method of Youngburg and Youngburg (12), using the phosphate procedure of Fiske and SubbaRow(13).

Results. In the first experiment, weight increases were similar in both groups, averaging 70% and 68% during 132 days for group A and B, respectively.

The rise in serum lipid components studied for both groups is shown in Table I. The sudden rise in the average of all lipids in group B on the 132nd day is due to a sudden inexplicable increase in the values of one animal. During the 20 day period, this animal showed a rise (which was rechecked on another day) of 749 mg in total cholesterol, 504 mg in cholesterol esters and 3.6 mg in lipid phosphorus, while the other rabbits in group B showed no significant rise. It would appear that under the conditions of this experiment, inositol almost completely inhibits the rise in serum lipids following cholesterol feeding, except in the case of one rabbit at 132 days.

The ester/total cholesterol ratio showed no significant change over the course of the experiment in either group, but the molar ratio of total cholesterol to lipid phosphorus increased more in group A (76%) than in group B (26%).

Table II shows that the results of the second experiment are qualitatively the same as in the first experiment. With this dietary regimen inositol was apparently not as effective in preventing a further rise in serum lipids. This may be due to the fact that the rabbits started at a higher lipid level than in the first experiment, that they received extra lipid (as salad oil) in their diet, or to the possibility that the amount of inositol required for inhibition may increase as the cholesterol intake increases.

Conclusions. From the above experiments, it would appear that inositol inhibits the expected rise in cholesterol and phospholipid levels in serum of rabbits on high cholesterol diets.

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Response of Pigs to Feeding of Vitamin B_{12}, Streptomycin and Sulfathalidine.* (19010)

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There are numerous reports in the literature of the increased growth response obtained with pigs fed vit. B_{12}, and various antibiotics. In the case of the antibiotics the reason for the growth stimulus is not fully understood, but is attributed to specific or selective bacteriostatic action on the gastrointestinal microflora. This being the case it might be expected that a similar growth stimulation might result from the feeding of certain sulfonamides. However, agreement in the literature on this point is lacking, the sulfonamides having been used in nutrition studies mainly as probable inhibitors of microorganisms responsible for vitamin synthesis.

Wahlstrom, Terrill, and Johnson(1) have reported that although phthalylsulfathiazole (sulfathalidine) reduced the number of coliform bacteria present in the feces of baby pigs fed a complete synthetic milk, the addition of 2% sulfathalidine to this diet did not significantly increase 8-week gains in body weight. Previously Lindley and Cunha(2), working with pigs 16 weeks of age which were fed a synthetic diet, observed that sulfathalidine fed at a level of 5% of the ration, proved to be very effective in decreasing intestinal synthesis of vitamins over a 6-week period and caused a decrease in daily gains of approximately 40%. These authors described a syndrome in pigs fed sulfathalidine which was prevented by the addition of biotin to the ration, and which was alleviated to a large extent by inositol.

Schultze(3) has reported the beneficial effect of feeding sulfathalidine to rats, with the evidence indirectly suggesting an alteration of the intestinal flora which favored the microbiological synthesis of vit. B_{12}. Dietrick, Monson, and Elvehjem(4) have described experiments with chicks fed a synthetic diet, in which the addition of 1% of succinylsulfathiazole (sulfasuxidine) produced a growth response which was enhanced by the addition of vit. B_{12}, when a high level of B complex vitamins was included in the diet. These authors point out that sulfasuxidine does not inhibit the growth response attributed to vit. B_{12}, if the level of the other B vitamins is adequate.

Experimental. In a preliminary experiment conducted by the authors it appeared that some growth response was obtained with weanling pigs weighing 30-35 lb, when 0.5% sulfasuxidine was added to a basal diet consisting of ground yellow corn, soybean oil meal, methionine, minerals, vit. A and D and riboflavin. The inclusion of methionine in this diet was suggested by the work of Bell and associates(5). An outstanding growth stimulation was also obtained when aureomycin was fed with this diet.

Subsequently in a series of tests with weanling pigs, designed to evaluate the growth-promoting effects of certain antibiotics and of commercial sources of vit. B_{12}, one group of pigs was fed the basal ration supplemented with 0.5% sulfathalidine. The pigs were kept indoors in pens with concrete floors and were fed in groups. The results are given in Table I. Results are also included for groups

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* Supported in part by a research grant by U. S. Industrial Chemicals, Inc., New York City.

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TABLE I. Effect of Vit. B_{12}, Streptomycin and Vit. B_{12}, and Sulfathalidine on Growth of Pigs.

Group No.	1 Basal[*]	2 Basal + .5% sulfathalidine[†]	3 Basal + B_{12}[‡]	4 Basal + B_{12}[‡] + streptomycin[§]
No. of pigs	4	6	5	5
Avg initial wt, lb	27.8	22.2	29	30.2
" final " "	173.8	196.8	194.2	211
Avg daily gain, first 7 wk, lb	.90	1.06	.93	1.09
Avg daily gain, 17 wk, lb	1.23	1.48[¶]	1.39[¶]	1.52[¶]

[*] Basal ration: ground yellow corn 63.60, soybean oil meal 34.25, methionine .15, salt .25, vit. A and D concentrate .15, B vit. mixture .60,[||] minerals 1.00.

[†] Contributed by Sharp and Dohme, Inc., Philadelphia, Pa.

[‡] APF Feed Supplement (U. S. Industrial Chemicals, Inc.) added to supply 1.05 mg B_{12} per 100 lb feed.

[§] 2 g per 100 lb feed.

[||] To supply 200 mg thiamine hydrochloride, 3 g niacin, 200 mg riboflavin, 1 g calcium pantothenate, 100 mg pyridoxine hydrochloride and 25 g choline per 100 lb feed.

[¶] Groups 2 and 4 significantly higher than groups 1 and 3, and group 3 significantly higher than group 1 at $P = .05$.

of pigs fed only the basal ration, and the basal ration supplemented with a commercial source of vit. B_{12}, and with streptomycin and B_{12}. Average daily gains are given for periods of 7 and 17 weeks.

A definite growth response was obtained, with the pigs fed sulfathalidine, which was of the same order as that obtained with the group fed the combination of streptomycin and B_{12}, and exceeded that for the group receiving only the B_{12} supplement. The basal diet used is one which the authors have used in a considerable number of experiments and which has proved very satisfactory for tests of this type with weanling pigs. Pigs fed this diet have consistently made good growth approximating 1.25 lb per day over an extended period. In our opinion the inclusion of B factors in ample amounts in this diet is very essential not only to support growth on the basal diet, but to permit the additional growth induced by supplements such as B_{12}, the antibiotics and in this instance sulfathalidine. While no direct evidence is presented in this report in support of this point of view, it has appeared that responses with the above named factors either are not obtained or are more limited, when the basal diet does not contain the B factors in ample amounts. Pigs fed this diet with the B factors included grow well, and have normal healthy skin and hair coats and show no evidence of any deficiency.

The results also point out that the growth stimulation resulting from B_{12}, streptomycin and sulfathalidine apparently was sustained throughout the entire experimental period of 17 weeks. The differences in growth as seen at the end of 17 weeks do not appear to be the result of growth stimulation in the early weeks of the experiment alone, but actually are more apparent with the progression in length of the feeding period.

Summary Increased growth of pigs fed for 17 weeks after weaning was obtained as the result of the addition of vit. B_{12}, streptomycin and B_{12}, or 0.5% sulfathalidine to a basal diet consisting of ground yellow corn, soybean oil meal, methionine, minerals and vitamins. Approximately the same growth was obtained by the feeding of the sulfathalidine, and the combination of B_{12} and streptomycin, the growth in both instances being greater than that obtained with the pigs with the addition of B_{12} alone. The increased growth obtained by all of the additions to the diet was sustained over the entire experimental period of 17 weeks.

NOTE: Since this paper was submitted for publication, Heidebrecht *et al.* (Okla. Agr. Exp. Sta. Miscell. Pub. No. MP22, June 1951) have reported increased growth and improvement of the general appearance of pigs fed sulfathalidine as well as certain antibiotics.

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Influence of Adreno-Cortical Extract, Cortisone and Desoxycorticosterone on Blood Levels and Urinary Excretion of Ascorbic Acid.*† (19011)

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Experimental evidence from these laboratories has been offered to show that adrenocortical hormones play an active role in the metabolism of ascorbic acid(1-3). In conditions of cold stress and in daily administration of ACTH, it has been found that the usual loss of ascorbic acid from the adrenals can be prevented greatly by adrenocortical hormones(4). Schaffenburg, Masson, and Corcoran(5) have recently called attention to the interrelationships between ascorbic acid and cortisone and ascorbic acid and desoxycorticosterone in disturbances of collagenous tissue. They have shown that, while cortisone will reduce the severity of scurvy in guinea pigs, desoxycorticosterone will increase the severity of disease in all of its aspects. Furthermore, they have reported that adrenal tissue from animals on a cortisone-ascorbic acid regime contained greater quantities of ascorbic acid than adrenal tissue taken from animals on ascorbic acid-desoxycorticosterone regime. They believe that the diseases of adrenal insufficiency and scurvy are similar.

In view of the very wide interest in the use of adreno-steroids in collagenous diseases and the fairly well established view that ascorbic acid is important in influencing metabolism and in maintaining integrity of collagenous tissue, it was thought well worthwhile and valuable to make a comparison of the adreno-steroids as they influence the metabolism of ascorbic acid. Such a study, we believe, may contribute to the understanding of the mechanism by which the adreno-steroids may be effective in certain collagenous diseases, and may define limits to which ascorbic acid is interrelated therein.

Methods. The following experiments were performed: 1. The plasma and cell ascorbic acid and 24-hour urinary excretion of ascorbic acid were studied in dogs on normal diet; dogs given ascorbic, 10 mg/kg daily, in addition to the normal diet; and dogs on normal diets given daily ascorbic acid as above and injected daily with one of the following adreno-steroids: adrenocortical extract, 5 dog units/kg; desoxycorticosterone, 2 mg/kg; and cortisone, 2 mg/kg. Eighteen daily experiments were performed on 3 dogs for each category, excepting the cortisone-ascorbic acid group. In this group, 12 daily experiments were performed. There were control dogs run with each experimental period, which covered 24 hours. Also each group was itself observed in control periods prior to the experimental period. 2. Four rats weighing an average of 225 g were placed in each of 5 metabolism cages and arranged on the following regimen for 4 days: group (A), con trol, normal diet; group (B), injected intramuscularly with 10 mg/100 g body weight of ascorbic acid daily; group (C), ascorbic acid as above plus 2 mg/100 g body weight desoxycorticosterone daily; group (D), ascorbic acid as above plus 5 dog units adrenocortical extract (Upjohn) per 100 g body weight of cortisone daily. Daily water intake-urine output records were kept. Ascorbic acid was determined on plasma and on blood cells(6)

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† Abstract of this work was presented before American Physiological Society, Columbus, O., Sept., 1950.

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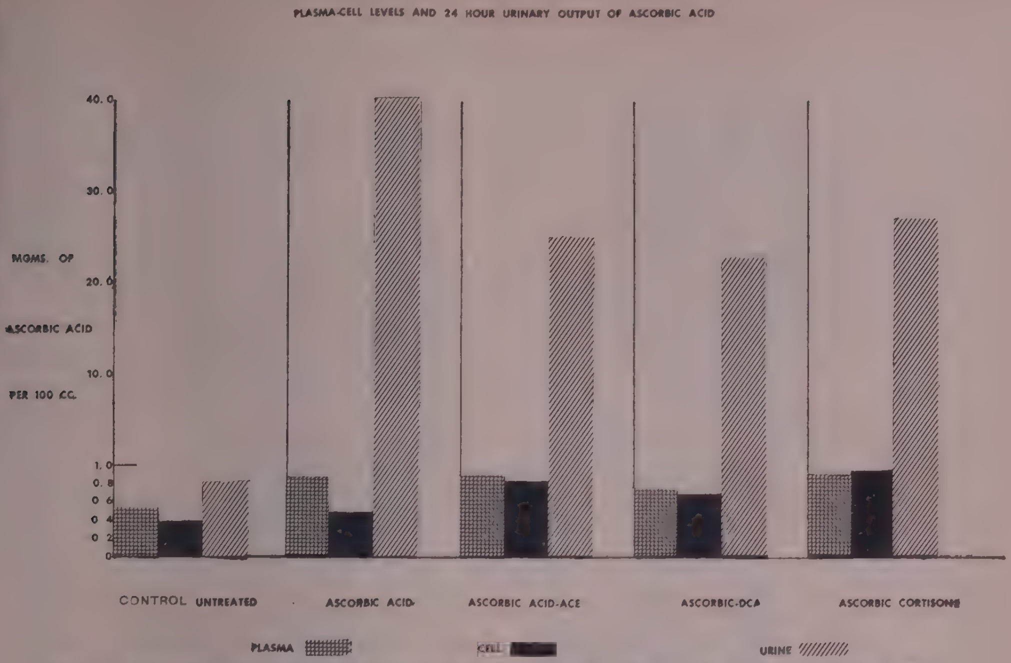

FIG. 1. Showing effects of adreno-steroids on plasma and cell ascorbic acid levels and on 24 hr urinary output of ascorbic acid in dogs. Values are mean values obtained from four experiments on each of three dogs.

according to the method of Mindlin and Butler(7). Two such experiments were performed involving a total of 40 rats. 3. From the above groups at sacrifice, the following tissues were removed from each animal and pooled for each category: Adrenals, liver, kidney, brain, skeletal muscle, tendon, fat and spleen. Homogenates of the pooled tissues were prepared in metaphosphoric acid for ascorbic acid determination.

Results. Fig. 1 is a representation of an average of 4 experiments on 3 dogs, showing changes in plasma and cell ascorbic acid and 24-hour urinary excretion of ascorbic acid under the regimen of ascorbic acid only, ascorbic acid plus adrenocortical extract, ascorbic acid plus desoxycorticosterone and ascorbic acid plus cortisone. Note that the plasma and cell ascorbic acid levels were increased by the adreno-steroids above the values seen where ascorbic acid only was administered. Note also that the increase in the plasma ascorbic acid where the adreno-steroids were administered along with ascorbic acid is not as striking as the increase in cell ascorbic acid. In the ascorbic acid-adrenocortical extract group the cell ascorbic acid increased 60% over the group receiving ascorbic acid only; while in the ascorbic acid-desoxycorticosterone group it increased 40%, and in the ascorbic acid-cortisone group, the ascorbic acid increased 80% above the group receiving only ascorbic acid. Regarding the 24-hour urinary excretion of ascorbic acid, it is of interest that the output of ascorbic acid was reduced 37% by adrenocortical extract; reduced by 42% by desoxycorticosterone, and in the cortisone group, the reduction was 30%, compared with the ascorbic acid alone group.

Fig. 2 presents the results of average values from rat experiments, showing the influence of the adreno-steroids on the 24-hour urinary excretion in rats. It can be observed that cortisone decreases the daily loss of ascorbic

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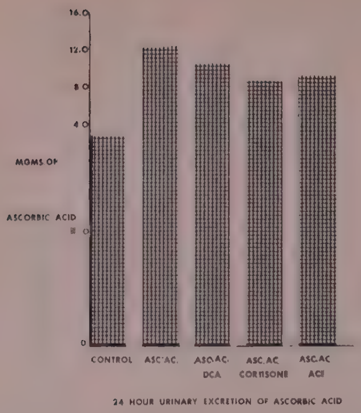

FIG. 2. Showing influence of adreno-steroids on 24-hr urinary excretion of ascorbic acid in rats. Values are representative of means of 8 animals in each category.

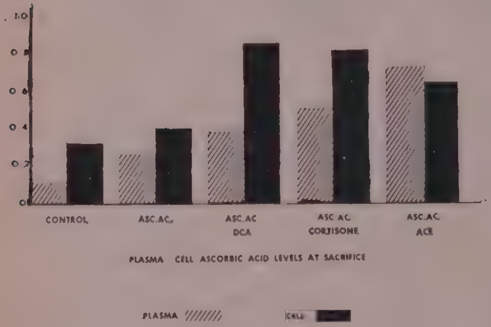

FIG. 3.

acid by 36%; adrenocortical extract by 23%; desoxycorticosterone by 15%. All values are compared with the group given ascorbic acid alone.

Fig. 3 presents data on ascorbic acid in plasma and in blood cells of rats at sacrifice, following 4 days on the respective regimen described. It is obvious that, as in the case of dogs, the adreno-steroids as a group increased the levels of ascorbic acid in blood. Note that desoxycorticosterone and cortisone are more effective in increasing cell ascorbic acid than adrenocortical extract, and that adrenocortical extract is more effective than either cortisone or desoxycorticosterone in building up plasma ascorbic acid.

Tissue distribution studies did not reveal any consistent trend regarding the influence of adreno-steroids on the deposition of ascorbic except at the adrenal gland. Here, desoxycorticosterone increased the ascorbic acid content of the gland by 10%; cortisone by 30%, and adrenocortical extract by 40%. In brain, those animals on ascorbic acid which received also adreno-steroids, showed a decrease of the ascorbic acid content of more than 100% below that of animals receiving ascorbic acid only. In muscle, adrenocortical extract and cortisone decreased ascorbic acid storage by 450% and 250%, respectively, below that of animals receiving ascorbic acid only. In fat, where the ascorbic acid content of the ascorbic acid treated group was surprisingly high (1.4 mg % on the average), cortisone and adrenocortical extract decreased the ascorbic acid levels 200 and 300%, respectively, but desoxycorticosterone increased the ascorbic acid by 50%. In tendon, the ascorbic acid treated group showed 10 times as much ascorbic acid as the untreated controls, while the cortisone-ascorbic acid and desoxycorticosterone-ascorbic acid group showed an increase of only 290% and the adrenocortical extract-ascorbic acid group showed an increase of 500%.

Discussion. The foregoing experiments show that adrenocortical extract, cortisone and desoxycorticosterone act to increase the plasma and cell ascorbic acid and to lower the urinary excretion of ascorbic acid. The influence of cortisone upon the permeability of the blood cells to ascorbic acid is in agreement with the works of others pointing out the value of cortisone in this connection(8,9). Cortisone is more effective in lowering urinary excretion of ascorbic acid than cortisone or adreno-cortical extract; although in rats cortisone controls the urinary output of ascorbic acid better than either of the above. The greater influence of adrenocortical extract and cortisone on storage of ascorbic acid in the adrenal gland than that of desoxycorticosterone is, in our opinion, significant. We have described experiments in another communication(10) showing that ascorbic acid storage

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at the adrenal gland is enhanced by adrenocortical extract. If adrenocortical function and ascorbic acid storage are related, then it is understandable that adrenocortical extract and cortisone would be more effective than desoxycorticosterone in ameliorating the symptoms of scurvy as shown by Schaffenburg, Masson, and Corcoran (referred to above). These workers point out that the symptoms of experimental scurvy and of adrenal insufficiency are similar. It is of interest in this connection that some workers have found that in ascorbic acid deficiency, the adrenocorticosteroid is markedly lowered (11), and that the symptoms of scurvy can be partially corrected with adrenocortical extract. It is of further interest that desoxycorticosterone will aggravate scurvy and other collagenous diseases. As we see the problem, it is not enough to raise the kidney threshold to ascorbic acid and to build up high levels in blood cells without at the same time increasing the storage of ascorbic acid at the adrenal gland. It is likely in this regard that desoxycorticosterone may differ from cortisone and adrenocortical extract in its influence on the course of collagenous diseases. This is a point, however, that should remain open until further work has been done. Indeed, Schaffenburg, Masson, and Corcoran have expressed the view that vit. C deficiency in arthritis of scorbutic animals leads to deficiency of carbohydrate-active cortico-steroids, the lack of which may underlie collagen degeneration. The results of this study and data from unpublished experiments lead us to agree in principle with the above work, although the mechanism is still unexplained. Certainly, we believe that adreno-cortical function is important in the maintenance of normal blood levels of ascorbic acid. On the other hand, it appears that normal adrenocortical function is dependent to some extent upon proper storage of ascorbic acid at the adrenal gland.

Summary and conclusion. (1) Experiments have been described comparing the effects of adrenocortical extract, desoxycorticosterone and cortisone on blood levels and urinary excretion of ascorbic acid in dogs and in rats. 2. The data show that all of the above substances reduce the urinary excretion of ascorbic acid. In this connection, cortisone is more effective in rats, while desoxycorticosterone is the most effective in dogs. 3. All the compounds caused increased plasma and cell ascorbic acid in dogs and in rats. 4. Inconsistent patterns were seen in the influence of the adreno-steroids on tissue distribution of ascorbic acid. In many instances, the adreno-steroids reduced markedly the tissue content of ascorbic acid. 5. Adrenocortical extract and cortisone were more effective than desoxycorticosterone on storage of ascorbic acid at the adrenal gland.

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Isolated Rat Liver Preparation. Bile Production and Other Basic Properties.*† (19012)

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The liver, more perhaps than any other organ, reflects in its structure and function changes impressed upon any part of the organism of which it is a part. Its close integration into the body economy, and its target position with respect to many drugs or diseases, render at once important, and difficult of achievement, an understanding of the functioning of this organ *per se*. The present paper is concerned with the description of an isolated liver preparation which retains a sufficient proportion of its normal physiological functions, and which survives long enough, to warrant study of its properties; it shows promise of permitting separation and analysis of many of the factors which affect the performance of this organ, an analysis of which is basic to the formulation of a usable concept of "liver function." In the past, perfusions of the isolated liver have been carried out in general as parts of biochemical investigations (1). Histological integrity of the tissue, bile production, and Kupffer cell activity were not studied, or were found to be poorly preserved after even short periods of perfusion. Furthermore, with one exception(2), survival of perfused liver tissue has been too short for many of the studies envisaged here.

The present preparation is based upon the following considerations: 1. The vascular tree of the liver is sensitive to perfusion pressures; unless sufficiently low inflow pressures are maintained at every moment some functions and structural integrity are soon lost(3). 2. The hepatic arterial supply is not essential for normal function of the rat nor even of the dog liver, provided adequate antibiotic levels are maintained in the blood supplied to the organ(4,5). 3. Survival of hepatic cells in organ culture is possible for periods of several days at least(2). 4. The heart-lung-liver preparation in the dog—contrast to saline perfused liver preparations—will produce bile, though none too well and not for very long(6). Thus, erythrocytes in the perfusate are, and innervation apparently is not, essential for bile production.

Methods. The perfusion medium is prepared in 3 separate solutions as follows:

A—2.5 g crystalline bovine plasma albumin,§ 37.5 mg $NaHCO_3$, 460 mg NaCl, 21 mg KCl, 6 mg $CaCl_2$, made up to 50 cc with distilled water.

B—4 cc 0.1 M Na_2HPO_4, 1 cc 0.1 M KH_2PO_4.

C—25 cc whole rat blood, containing 0.5 mg of heparin. Solutions A and B are mixed and equilibrated in the perfusion pump with 95% O_2 and 5% CO_2 mixture. C is then added. To secure constant glucose and antibiotic levels 0.012 cc/min. of the following solution are infused throughout each experi-

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* Presented in part before the Am. Soc. Exp. Pharm. and Ther., Fall, 1950, cf. J. P. E. T., 101, 4, 1951.

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‡ Present address: U. S. Naval Radiological Defense Laboratory (Pharmacology Section), San Francisco, Calif.

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§ The authors wish to thank Dr. Edwin E. Hayes of Armour and Co. for making this material available in the amounts required for this work.

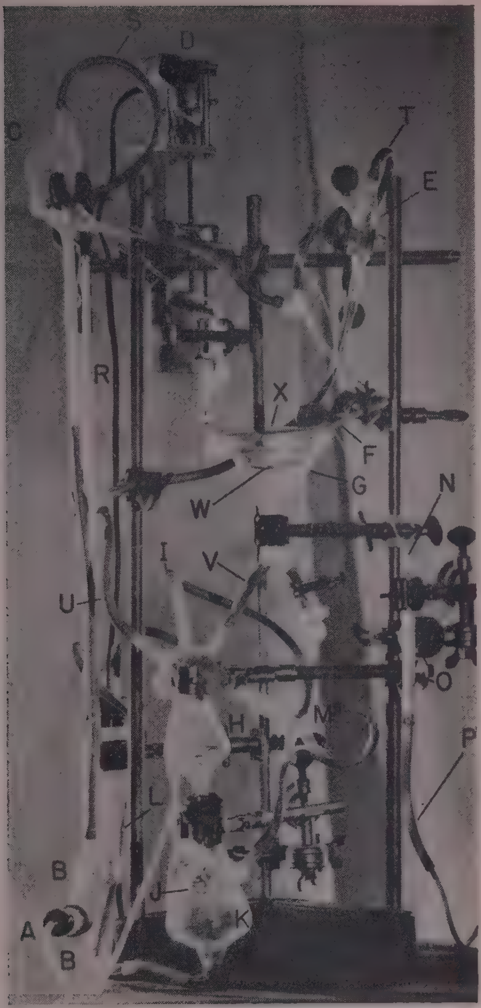

FIG. 1. Apparatus for isolated liver experiments. Perfusate path-K-B-A-B (valves and pulsating rubber finger pump)-C-E-(pressure control heads—open to atmosphere through gauze filters at ends of S and T)-U-H (falling film oxygenator)-J (nylon bag filter)-K. Supply to the liver tapped off between C and E through F. Oxygen-CO_2 passes through saline bubbler L, manifold, and trap M, to line I entering H (gas escapes through capped vent in back of H), also to reservoir K. Continuous infusions are delivered by pump D, catheter, and needle to E. For other letters and details of operation see text.

ment:

D—25 mg terramycin hydrochloride (Pfizer & Co.), 50 mg dextrose, 500 mg crystalline bovine plasma albumin, 92 mg NaCl, 4.2 mg KCl, 1.2 mg $CaCl_2$, made up to 10 cc with distilled water. Where desired the dye sodium tetrabromphenolphthalein disulfonate (BSP)¶ (3 mg/cc) or other drugs are added to this solution. The solutions are sterilized by filtration. Blood is obtained under aseptic conditions by heart puncture.

The perfusion pump used is a modification of those described in references(7) and takes into account the first point made above as well as the reduction in size allowed by the use of rat tissues. The general arrangement shown in Fig. 1 is operated inside a constant temperature cabinet. The circuit consists of a perfusate reservoir K, a modified Carrel-Lindbergh pump, BAB, a pressure and pulse pressure regulating system C-E (note the flaring connections found important for erythrocyte preservation), a falling film aerator H, and a return through a nylon bag filter J to the reservoir. The blood supply to the organ is tapped from the pressure regulated supply between C and E through F, and returns to the system via funnel G and aerator H. The gas mixture is bubbled through saline in the saturator L before being supplied to the oxygenator and the reservoir via M and I. All glass parts of this system except H are coated with silicone‖ for better blood preservation. Sterilization of all parts except A is carried out by autoclaving the assembled system (15 lb, 20 min.). A is cleaned, kept under Zephiran Cl (1:500) for 3 hours or more, flushed with saline and connected into the system with aseptic precautions. Antibiotics and other materials supplied to this circuit continuously are administered by the continuous infusion pump D. The pulsating air stream which operates pump A is supplied by a mechanical pump (*e.g.* the Palmer Ideal Respiration Pump) at about 80 strokes per minute and with such pulsation amplitude as to roughly equalize the perfusate streams flowing through F-V and bypass U; the absolute flow through C may vary within wide limits without affecting the performance of the perfused liver. Blood flow through the organ can be determined by clamping V and noting the time elapsing before the fluid level in G reaches a set mark (corresponding to 10 cc). Bile is collected in a Wintrobe hematocrit tube O; bile flow rates are measured as rates of rise of the fluid column in this tube.

¶ The authors wish to thank Dr. H. B. Dunning of Hynson, Westcott and Dunning for supplying the BSP used in this work.

7. a. Werthessen, W. T., *Endocrinology,* 1949, v44, 109. b. Hechter, O., *Endocrinology,* 1948, v42, 285.

‖ The authors are indebted to the General Electric Co., Schenectady, for supplying "Dry Film 9987", the organo silicone used in this work.

Non-fasted Sprague-Dawley rats were used as blood and liver donors. Livers were removed with sterile technic under sodium pentobarbital anesthesia (39 mg/kg intraperitoneally) after insertion of a catheter** into the bile duct, a cannula into the vena porta, and an outflow catheter (3″, 3 mm i.d.—this at once assures free flow, and negative pressures at the hepatic veins) into the thoracic vena cava. The organ is placed diaphragmatic side down into a silicone lined porcelain dish (W) with a 5 mm hole in the bottom through which the return cannula in the vena cava is passed. Peculiar aspects to be noted about the operative technic are the following: (a) The esophagus is resected close to the stomach; this permits retraction of the esophagus into the thorax during the critical last stage of removal of the liver and speedier transfer to the pump. (b) Heparin (0.2 mg) is administered 2 minutes before occlusion of the vena cava; experience has shown that this results in more satisfactory clearing and better survival of the organ. (c) Circulation to the liver is seriously reduced and eventually totally stopped from the moment the portal vein is occluded to the moment the liver is inserted into the perfusion stream; it is important that this ischemic interval be kept as short as possible—3 to 5 minutes is considered satisfactory in this laboratory. Performance and survival are seriously reduced if ischemia exceeds 10 minutes. Samples taken for analysis include bile, drawn from the collecting tube every 15 minutes, or for special purposes collected in fresh tubes for 5 or 10 minute intervals; blood drawn at U for mixed, and at V for hepatic venous samples; and liver specimens obtained at "biopsy" or terminally. Determinations include total plasma proteins (8), blood glucose(9), blood and bile bromsulfalein contents(10), and hematocrits. Bile total solid content is determined by weighing drops of bile placed on polystyrene film,†† drying over silica gel, and reweighing. Volumes of 5 microliters suffice for this determination. Histological examinations here reported were made on formalin fixed sections, paraffin embedded and stained with hematoxylin and eosin.

Results. Isolated livers perfused under the conditions described, quickly recover from the operative ischemia. Within 3 minutes the organs appear uniformly pink. Simultaneously bile formation is resumed. Fig. 2 shows changes in rate of bile formation which are representative of the majority of experiments: an initial recovery period is followed by a relatively brief period of rapid bile flow which terminates after 2 to 3 hours and is succeeded by a prolonged period of less rapid but steady bile production. This phase may last from 5 to 12 hours, and, in turn, leads into the terminal phase of dwindling bile flow. Paralleling the bile flow are changes in appearance of the organ: the first phase is marked by resumption of blood flow and of a uniform, pink and healthy appearance. This is preserved through the succeeding two phases. The terminal phase is accompanied by the development of an increasing number of punctate hemorrhages which may eventually spread over most of the surface of the organ, and by increasing congestion and impaired circulation. Microscopically the first phases are marked by excellent preservation of the tissue, so much so that it is not readily distin-

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** The authors are indebted to the Irvington Varnish and Insulating Co. for supplying the Polystyrene plastic tubing (0.025″ I.D., 0.004″ wall thickness) employed in this work.

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9. Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, v83, 115.

10. Brauer, R. W., and Pessotti, R. L., *Am. J. Physiol.*, 1950, v163, 565.

†† The authors are indebted to Dielect, Perth Amboy, N. J. for supplying this material 0.0008″ thick.

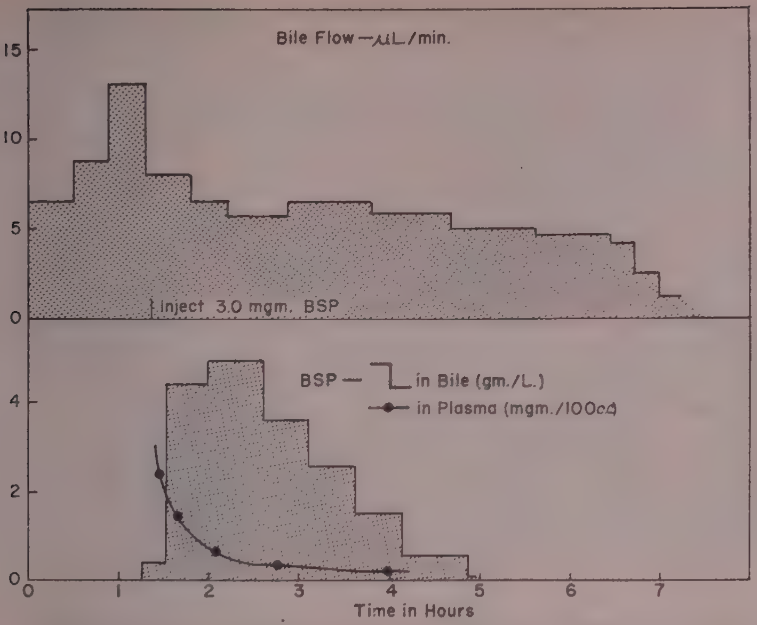

FIG. 2. Representative course of bile production in isolated liver showing four phases of activity, and course of plasma and bile BSP concentration after single BSP injection (Rat 50-50 ♂ 220 g).

guished from freshly cut liver of healthy animals (Fig. 3). Occasionally, cell free spaces are seen, especially around the central vein; these may possibly denote early edema. During the terminal phase, in addition to extravasation and congestion, regions of central necrosis may appear. With improved media there has been some tendency to find relatively intact histology even after cessation of bile flow.

Bile production by the isolated liver is affected by a variety of factors. Some of these, notably perfusion pressure, oxygen tension, pH of the perfusate, and temperature, will be discussed in greater detail in a subsequent communication dealing with the mechanism of bile production. For present purposes it may suffice to indicate that the following is a satisfactory set of operating conditions: Temperature, 37°C; gas mixture, 95% O_2—5% CO_2; perfusate pressure, 21.5 cm H_2O; pH, 7.4. None of these conditions are critical since they appear close to the optima for each variable.

Survival of the isolated liver as indicated by the period of bile production is positively correlated with the body weight of the donor. Thus, rats weighing between 160 and 200 g, yielded livers surviving for a mean period of 4 hours (3.5-4.5 hr), between 200 and 233 g, for 6.6 hours (5.0-7.5 hr), between 233 to 266 g for 8.3 hours (5.3-13 hr), and those between 266 and 300 g for 10.4 hours (8-17 hr). In 3 animals starved for 24 hours before removal of the livers, survival of the isolated organs was significantly shorter than for non-fasted animals of the same weight (290 g, 3.5 hr; 240 g, 3 hr; 240 g, 3.5 hr). On the other hand, enrichment of the medium described above with amino acids and vitamins (to approximate White's(11) medium) resulted in series of exceptionally long survivals ranging from 15 to 30 hours.

Bile secretion rates, concentrations, and pressures are summarized in items 7 and 8 of Table I, showing values for the steady bile flow phase. During the 2nd—the rapid flow phase—both flow rates and solid concentra-

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11. White, P. R., *Growth*, 1946, v10, 231.

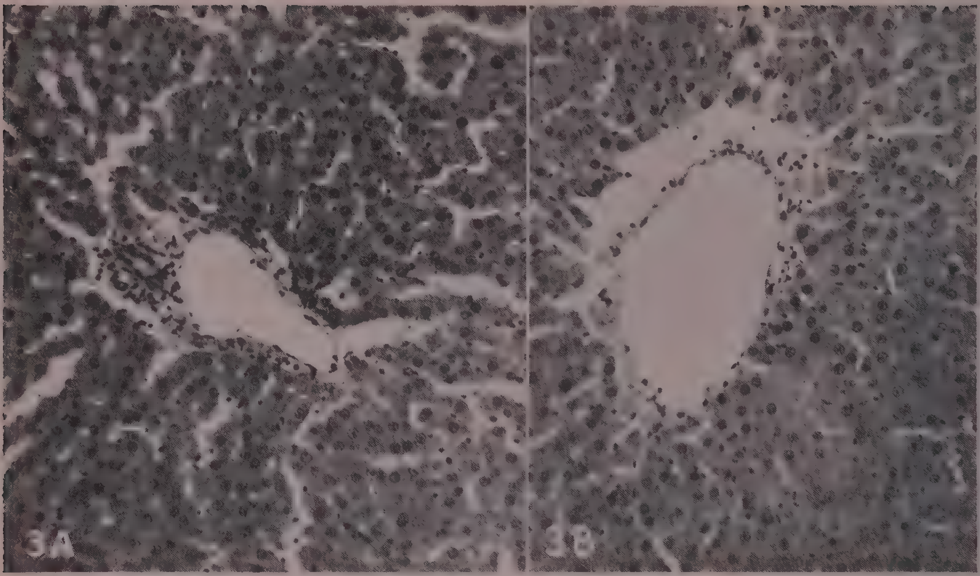

FIG. 3. Liver Rat 50-55 ♀ 300 g, after 24 hr in the perfusion pump. (a) Periportal area showing well preserved hepatic cords with slightly dilated sinusoids. 160 ×. (b) Central vein showing intact hepatic cords and sinusoids. Occasional necrotic cells are found adjacent to the vein. The clear perivascular area is typical and may represent edema. 160 ×.

tions average slightly higher, namely in males 9.4 ± 0.61 μL/min. and 3.4 ± 0.4%, and for females 9.7 ± 0.71 μL/min. and 5.5 ± 0.38%.

Bromsulfalein is extracted from the perfusate by the isolated liver not only after a single injection, but also continuously during infusions. Fig. 2 shows the exponential decay of plasma BSP levels seen in about half of the experiments (a flattened curve such as is seen in infants and in certain types of liver injury is found in the other half); the course of bile BSP concentrations shown is typical of all preparations. Steady plasma and bile BSP levels can be reached in continuous infusion studies (see Table I, last two items). Bromsulfalein is excreted by the isolated liver in at least 3 separable components only one of which, accounting for about 20% of the dye recovered from bile, is indistinguishable from BSP(12).

The reticulo-endothelial system of the isolated liver remains functional; thus, India ink is quickly removed from the perfusate, and numerous engorged Kupffer cells can be found in sections of livers so treated (Fig. 4). Bromsulfalein extraction from the perfusate is reduced by India ink injection as shown in Fig. 5; perfusate BSP concentrations during continuous BSP infusion increase to higher plateaus after India ink administration. Bile flow decreases somewhat but biliary BSP concentrations are not readily affected.

Table I summarizes the performance of the isolated liver in the steady bile flow phase together with some of the relevant operating conditions. For purposes of discussion, comparison values for rat livers *in situ* in intact animals are included.

Discussion. The basic prerequisite for an isolated liver preparation that is to aid the understanding of the intact organism, is that all elements of the tissue must be maintained in a functional state. The most important result of the work here reported has been the demonstration that the isolated liver can form bile, and that bile flow can be maintained for extended periods of time. Conditions which were found compatible with bile formation also could be shown to result in preservation

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12. Brauer, R. W., Krebs, J. S., and Pessotti, R. L., *Fed. Proc.*, 1950, v9, 259.

TABLE I. Conditions and Performance of Isolated Liver During the Third (Steady Bile Flow) Phase, with Reference Data for the Liver *in situ*.

Description		Isolated liver	Liver, *in situ*
Body wt—g		275 ± 8.9 (30)*	250–310
Plasma vol—cc		72.1 ± 1.27 (30)	9.6–11.8 †
Hematocrit—%		11.3 ± .34 (27)	47 ± .41‡
Plasma protein—g/100 cc		4.8 ± .19 (25)	5.6 ± .4 ‡
Perfusate flow through liver—cc/min		93 ± 5.8 (20)	8–12 §
Glucose in perfusate—mg/100 cc		210 ± 13.7 (18)	115–140 ‡
Bile flow—μL/min	M	6.4 ± .60 (15)	17.3 ± 1.5 ‡
	F	5.1 ± .54 (14)	12 ± .6 ‡
Bile solids—g/100 cc	M	2.7 ± .2 (9)	3.2 ± .33‡
	F	3.6 ± .3 (10)	5.2 ± .29‡
Secretion pressure—cm bile		14.6 ± .82 (8)	20.3 ± 1.1 ‡
Time to remove 50% of a single injection of BSP from circulation—min		17 ± 2.5 (6)	5.6 ± .5 ‖
BSP in plasma (cont. infusion .2 mg/kg/min) —mg/L		24.7 ± 4.7 (6)	17.4 ± 3.1 ‡
BSP in bile (cont. infusion .2 mg/kg/min)—g/L		5.7 ± .59 (14)	4.1 ± .42‡

* No. in parentheses indicate No. of experiments.

† Calculated from data of reference 15.

‡ Data from reference 16.

§ In the absence of data for the rat the data of 17 for blood flow per g liver for man and dog have been used to calculate a reasonable range of values. Actual values are probably somewhat larger in the rat.

‖ Data from reference 3.

of the macroscopic and microscopic anatomy of the tissue, in the maintenance of functional Kupffer cells, and of continued detoxication activity, at least as indicated by the excretion of modified forms of BSP. Thus, evidence is on hand that the above basic condition is met by the preparation here described. The second key question to be answered for such a preparation concerns the "normalcy" of operating conditions and behavior. Table I shows some quantitative comparisons between the present preparation and rat livers *in situ* in intact animals. Items 3 and 5 probably represent the most important differences between the conditions to which a liver is exposed *in situ* and in perfusion. The low hematocrit values are useful in prolonged perfusions, to prevent sludging and liver edema, and to protect erythrocytes against mechanical destruction. Under these conditions high flow rates are required to supply adequate oxygen to the tissue; below 60 cc/min. bile flow and survival alike are impaired. The value of 90 cc/min. is below destructive pressure and flow levels and has been found satisfactory for most studies. Item 9—a key characteristic of BSP extraction curves after single dye injections—probably illustrates one of the few situations where lower perfusate flow rates would result in a closer approach to normal; the flat extraction curves probably result from imperfect utilization of the multi-plate extractor mechanism of the liver at the high throughput rates (3). Items 10 and 11 show that BSP handling *per se* is not seriously impaired in the isolated liver. Items 7 and 8 show that bile

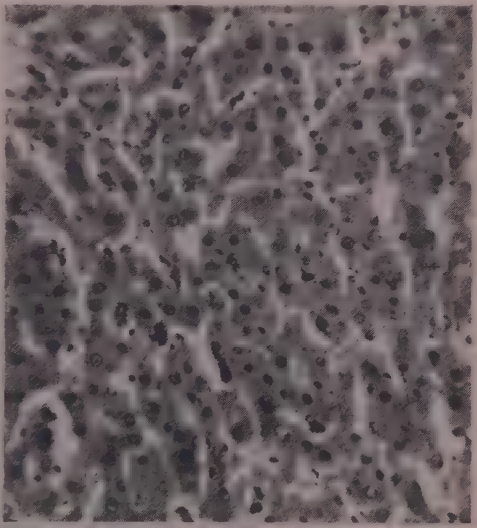

FIG. 4. Rat 59-59, ♀, 338 g. Section of liver six hr after India ink administration showing reticuloendothelial cells containing phagocytized India ink particles. 200 ×.

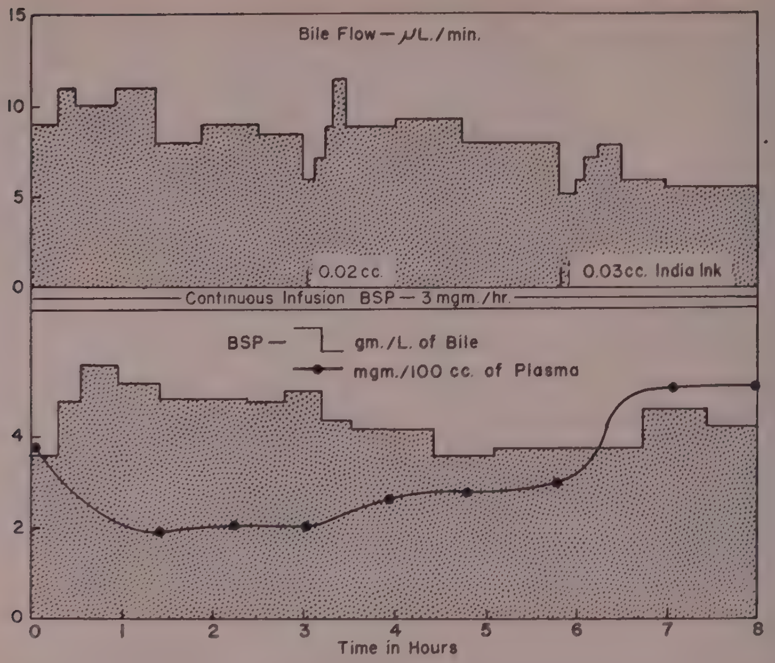

FIG. 5. The effect of India ink on bile flow, BSP uptake and excretion. Rat 50-59, ♀, 338 g.

production by the perfused liver is quantitatively comparable to that in the intact animal. This is especially evident if one recalls that optimal bile production occurs during phase 2 and not during phase 3 upon which Table I is based. The transition between these phases may be due to depletion of circulating bile acids caused by the lack of entero-hepatic circulation in the perfusion system, or to biochemical changes similar to these described by Lundsgaard(13). The glucose and total protein levels maintained (Items 4 and 6) are convenient but non-critical under present operating conditions.

It is felt that the mechanism and procedure here described are such that mechanical factors no longer control the duration of an experiment. All evidence at this time points to depletion of essential tissue components as the factor resulting in transition from phase 3 to phase 4: the relation between body weight of donor and liver survival, the effect of preoperative starvation, and the effect of the addition of nutrients alike point in this direction.

The present preparation is primarily designed for the study of bile formation, of the vascular reactions, and of the excretory mechanisms of the isolated liver. Extended survivals resulting from improvements in procedure should make the perfused liver a useful tool in the study of hepatotoxic agents, of reticulo-endothelial cell turnover and activity, and of nutrition and depletion of the liver.

Summary. (1) It has been shown that the isolated, perfused rat liver can be maintained under conditions which permit the continuous production of bile for periods up to 25 hours. (2) Livers thus maintained preserve an apparently normal histology up to the terminal

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17. Myers, J. D., *J. Clin. Invest.*, 1947, v26, 1130; and Grab, H., Janssen, S., and Rein, H. L., *Z. f. Biol.*, 1929, v89, 324.

phase. (3) The reticulo-endothelial cells of such perfused livers retain their ability to remove particulate matter from the perfusate stream. (4) Bromsulfalein is extracted from the perfusate by the isolated liver, and is re-excreted into the bile in high concentration and in several separable components, analogous to those found in the intact animal. (5) Data have been presented to permit a comparison of the conditions of operation and the performance of the isolated rat liver, and the liver *in situ* in the intact animal. (6) Details of the method of preparation, the perfusion pump, and the perfusion media have been presented, together with specific data concerning the course of typical experiments. (7) The range of applicability of the preparation in its present state of development and directions of further exploration have been discussed.

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Nucleic Acid Changes in Rat Nerve Tissue After Parenteral Administration of Vitamin B_{12}. (19013)

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The beneficial effects of vit. B_{12} in treating nutritional anemias(1,2) and neurological disturbances(3,4) have been demonstrated. The biochemical mechanisms and the cytological localization of the activity of this vitamin as yet are not fully known. Facilitation of the formation of ribonucleic acid in liver cells protected by large doses of vit. B_{12} against depletion by poisoning with carbon tetrachloride has been reported by Koch-Weser and Popper(5). On the assumption that the intensity of the basophilic reaction indicates the quantity of nucleoprotein present, Stern, Taylor, and Russell(6) studied weanling rat livers stained with methylene blue. These investigators found that there was almost no basophilia in rats on a diet containing soybean oil meal as the only protein source. The addition of vit. B_{12} to this diet produced a decided liver basophilia. Liver extract gave a slightly greater response. In 1950, after our work had begun, Seigel and Worley(7), using basophilia as an index of the presence of nucleic acids, confirmed the results of Stern *et al.* A nucleic acid abnormality in bone marrow cells due to pernicious anemia also has been corrected by local injection of vit. B_{12}(8). The present investigation has been undertaken to determine whether parenteral administration of B_{12} produces definite cytochemical changes in the nucleic acids in nerve tissue in rats. Several stains were used to visualize any possible changes in basophilia, total nucleic acids, pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA).

Materials and methods. Two experiments were carried out. Table I contains a summary of the dietary groups and the quantities of vit. B_{12}* administered. Food consumption records were kept daily and the animals weighed every other day. The basal diet was

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* The vit. B_{12} used in this study was furnished through the courtesy of Mr. A. W. Veazey of Merck & Co.

TABLE I. Summary of Experimental Groups and Quantities of Vit. B_{12} Administered Subcutaneously.

Exp.	Dietary groups	No. of rats	Parenteral B_{12}
1*	Chow diet†	4	Control
	” ”	4	.25 μg B_{12} dieb. alt. 20 days
	Basal ” ‡	4	Control
	” ”	4	.25 μg B_{12} dieb. alt. 20 days
2	Chow diet + .5% sulfa	4	Control
	” ” ” 2% ”	4	”
	Basal ” ” ” ”	3	”
	” ” ” ” ”	1	2 μg B_{12} dieb. alt. 4 days; 4 μg B_{12} dieb. alt. 6 days; last dose 10th day 7 μg B_{12}
	” ” ” .5% ”	2	

* The diets of all the rats in Exp. 1 were supplemented with .5% sulfa on 13th day of exp.

† Purina laboratory chow.

‡ Alcohol extracted casein 18, sucrose 68, corn oil 1, cellu flour 2, salt mixtures 3A 2 and 3B 2(9), vit. B complex mixture 5(9), and fat soluble vitamin mixture 2%(9).

deficient in vit. B_{12}, folic acid and para-aminobenzoic acid (PABA). By the 13th day of the first experiment the weanling rats on chow diet plus B_{12} were gaining weight more rapidly than those on unsupplemented chow diet. Those on the basal diet weighed slightly more than those on the basal plus B_{12}. In order to prevent possible synthesis of B_{12} due to intestinal bacteria, the diets of all the animals were supplemented with 0.5% sulfaguanidine (sulfa), to alter the intestinal flora. Growth rates were lessened for all groups. The discrepancy between the rats on basal diet and those on basal plus B_{12} was increased. All of these animals were sacrificed on the 20th day of the experiment. The 9-week-old rats† used in experiment 2 had been on a diet supplemented with either 0.5% sulfa or 2.0% sulfa, but deficient in vit. B_{12}, folic acid, PABA and vit. K for 6 weeks before we received them. At that time a group of 4 animals on chow diet plus 0.5% sulfa and another group of 4 on chow plus 2.0% sulfa were sacrificed. The remaining rats were divided into 2 groups and placed on the basal diet deficient in vit. B_{12}, PABA and folic acid supplemented with sulfa. The starting dosage of vit. B_{12} for the one animal on 2.0% sulfa and the 2 on 0.5% sulfa was 2 μg every other day for 4 days. Since these already stunted rats showed no growth response they were given 4 μg of B_{12} every other day for a total of 3 injections. On the 10th day they were each given 7 μg of B_{12} and sacrificed about 5 hours later along with the 3 rats which had been retained on the deficient diet plus 2.0% sulfa without parenteral B_{12}. Rats from both experiments were killed by a severe blow on the head. Tissues were placed immediately in Zenker's fixative and subsequently paraffin embedded. 10 μ sections of spinal cord, autonomic ganglia (cervical trunk) and liver from all animals have been examined.

Four stains were used on all tissues: 1. Hematoxylin and eosin for general basophilia. 2. A modification of Turchini's 9-methyl-2, 6, 7-trihydroxy-3-fluorone for selective staining of both PNA and DNA(10). 3. Feulgen reaction(11). 4. Einarsson's gallocyanin-chrome alum. This combination of stains made possible an examination of the cellular basophilia as well as of the PNA and DNA collectively and separately. Digestion of liver, spinal cord and autonomic ganglion sections with ribonuclease (Worthington) provided for a qualitative examination of the Turchini and Einarsson stains for PNA. Sections were placed in ribonuclease, 1.0 mg per ml of McIlvaine's buffer at pH 6.85 for 3 hours at 60°C. Control sections from the same block of tissue were placed in the buffered solution without the enzyme for the same time period.

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† Medical students demonstrated avitaminoses with these rats as a part of their biochemistry course. Dr. E. A. Doisy, Jr., who supplied us with animals for the first experiment, was also kind enough to allow us the use of these rats.

10. Backlar, B., and Alexander, W. F., to be published.

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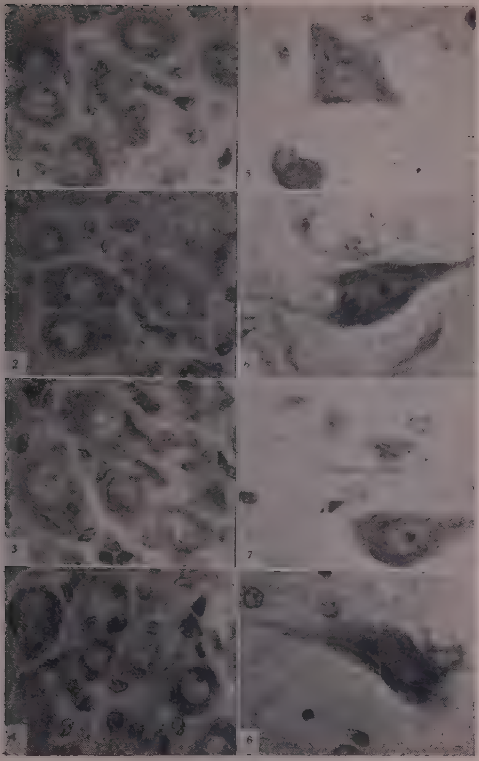
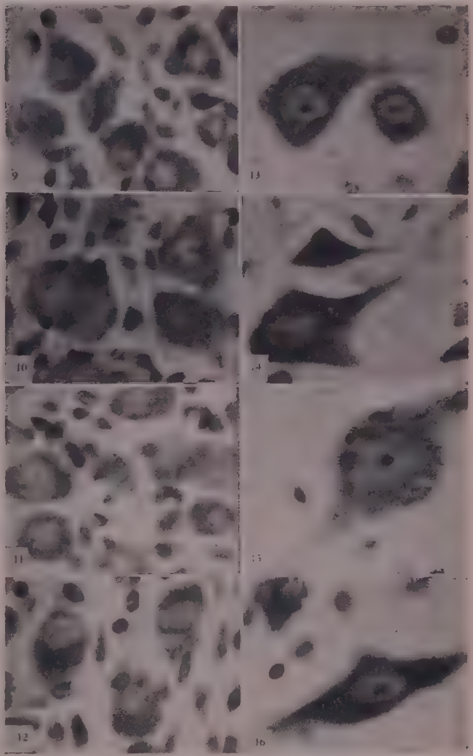


Turchini stain
FIG. 1. Autonomic ganglion, rat, chow diet.
FIG. 2. Autonomic ganglion, rat, chow diet + parenteral B_{12}.
FIG. 3. Autonomic ganglion, rat, basal diet.
FIG. 4. Autonomic ganglion, rat, basal diet + parenteral B_{12}.
FIG. 5. Anterior horn cells, rat, chow diet.
FIG. 6. Anterior horn cells, rat, chow diet + parenteral B_{12}.
FIG. 7. Anterior horn cells, rat, basal diet.
FIG. 8. Anterior horn cells, rat, basal diet + parenteral B_{12}.

The almost complete loss of cytoplasmic and nucleolar stain by the enzyme treated sections and the presence of the Turchini and Einarsson stains at the sites of PNA in the buffer treated sections is an indication of the qualitative reliability of these stains.

Results. There were no significant differences in the staining reactions when the tissues of one animal of a group were compared with the same tissues of other animals in the same group. There was a constant observable difference in the staining reaction when the same tissues from the various groups were compared, but no differences among animals within a group.

Our findings that staining with hematoxylin and eosin showed enhanced basophilia in the tissues studied in rats which had received B_{12} over those which had not, confirmed the results of previous workers(6,7). Because of the differentiation procedure involved in staining and because we are not convinced that basophilia, *per se,* is a positive indication of nucleic acid, these results were not considered significant. The Turchini stain produced consistent results. The demonstration of PNA



Einarsson stain
FIG. 9. Autonomic ganglion, rat, chow diet.
FIG. 10. Autonomic ganglion, rat, chow diet + parenteral B_{12}.
FIG. 11. Autonomic ganglion, rat, basal diet.
FIG. 12. Autonomic ganglion, rat, basal diet + parenteral B_{12}.
FIG. 13. Anterior horn cells, rat, chow diet.
FIG. 14. Anterior horn cells, rat, chow diet + parenteral B_{12}.
FIG. 15. Anterior horn cells, rat, basal diet.
FIG. 16. Anterior horn cells, rat, basal diet + parenteral B_{12}.

was clear cut. The tissues of the animals receiving B_{12} always showed more PNA than those of the controls (Fig. 1-8). Liver, anterior horn cells and autonomic ganglion cells all showed an increase in PNA. Definitive DNA changes in tissues stained by this method could not be observed although the localization, by color, was obvious. The presence of nuclear PNA was too distracting for an objective analysis of a cell's DNA content. Staining of these same tissues by the Einarsson method confirmed the results obtained through the Turchini procedure (Fig. 9-16). Since there is no color differentiation between PNA and DNA, attention was focused primarily upon the cytoplasm. Here one noted clear cut differences between preparations from B_{12} treated and those from control animals. Tissues of B_{12} treated rats always showed more PNA than the controls. The Feulgen stain indicated the presence of DNA. The same histological picture was seen, both quantitatively and qualitatively, for spinal cord and autonomic ganglia of all the animals. Preparations of liver showed a consistent, although slight, rise in DNA content in B_{12} treated rats. The results of the second experiment confirmed those of the first.

Summary. These preliminary results support the contention that vit. B_{12} is related to the genesis of ribonucleoproteins in rat tissues. Weanling and immature rats were placed in 2 dietary groups, chow and basal, the latter dietary group deficient in vit. B_{12}, folic acid and PABA. One-half of each group received parenteral B_{12} every other day. Liver, spinal cord and cervical sympathetic ganglia were examined histologically for changes in pentose and desoxypentose nucleic acids. These 3 tissues from animals which received vit. B_{12} showed more PNA than the controls. No consistent observable difference was seen in spinal cord and cervical sympathetic ganglia for the DNA as visualized by the Feulgen stain. There was a slight increase in the intensity of the Feulgen stain in hepatic cell nuclei from rats which had received vit. B_{12} over the controls.

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Studies on a Proteolytic Enzyme System of the Blood. IV. Activation of Profibrinolysin by Serum Fibrinolysokinase.* (19014)

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Recent studies in this laboratory concerning the activation of profibrinolysin (plasminogen) by fibrinolysokinases of bacterial(1,2) and tissue(3) origin have included observations which suggest that a kinase is also involved in the so-called "spontaneous" activation of serum profibrinolysin. Active fibrinolysin (plasmin) although not present in normal whole serum, has long been known to appear in certain serum fractions(4,5) and in whole serum after treatment with chloroform (6). Our working theory of the interactions of the various components of the fibrinolytic enzyme system of dog serum is:

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$$\begin{tikzcd}
& \text{Fibrinolysokinase} \arrow[d] & & \text{Antifibrinolysokinase} \arrow[ll, dashed] \\
\text{Profibrinolysin} \arrow[rr] & {} & \text{Fibrinolysin} \arrow[d] & \text{Antifibrinolysin} \arrow[l, dashed] \\
\text{Fibrin (other proteins)} \arrow[rrr] & & {} & \text{Protein fragments}
\end{tikzcd}$$

Profibrinolysin, the inactive enzyme precursor, is present in normal serum and may be converted to the active enzyme, fibrinolysin, by the action of a fibrinolysokinase. Fibrinolysokinase activity has been found in certain bacterial products(7-11) and certain tissues (3,12-16). Antifibrinolysin has been widely studied(17-20) and shown to be a normal serum component which inhibits the action of fibrinolysin. Antifibrinolysokinase has not been isolated and studied, but indirect evidence suggests that one or more such inhibitors (*e.g.* antistreptokinase)(7,21) may be present in serum and seem to be distinct from antifibrinolysin.

The experiments presented in this paper were designed to demonstrate the existence of a fibrinolysokinase in normal *serum* and to show that it is independent of cellular elements. All experiments concern dog serum or its fractions.

Methods. Previous studies have described the preparation of borate buffer, pH = 7.7 (20), staphylokinase(2) and our standard serum profibrinolysin preparation, Prolysin (3) (see below, Exp. III), and the determination of fibrinolysin and antifibrinolysin concentrations(20). Profibrinolysin content(2) of a serum fraction, free from antifibrinolysin, was determined by incubating it with 2% staphylokinase at 5°C for 2 weeks and titering the fibrinolysin content at intervals until it is stable.

Results. I. *Is a fibrinolysokinase, released from the cellular elements of the blood, responsible for the "spontaneous" activation of profibrinolysin?* The development of fibrinolytic activity in chloroformed whole serum was used as a test system.

Two lots of arterial blood, obtained from the same dog through a siliconed cannula were each mixed with one-tenth volume of 0.1 M sodium oxalate. Lot I was mixed in a glass container and subsequently centrifuged at 320 g for 15 minutes. Smear of this slightly turbid plasma showed many platelets and occasional erythrocytes and leucocytes. The second lot of blood was collected into a chilled siliconed container, centrifuged for 30 minutes at 2000 g, the supernatant plasma transferred to a cellulose nitrate tube with stainless steel collar and centrifuged an additional 60 minutes at 42,000 g. Smear of this clear plasma showed no cellular elements. Recalcification times were respectively: Lot I, 50 seconds; Lot II, 275 seconds. Sera were prepared from each lot of plasma in two ways: (a) by recalcification: IA, IIA and (b) by addition of thrombin (Upjohn): IB, IIB. The sera were treated with chloroform by shaking vigorously for 2 minutes with ½ volume of chloroform and centrifuging 15 minutes at 720 g to remove excess chloroform and part of the denatured protein. The chloroformed sera and control untreated sera were incubated over a 3-weeks' period at 4°-6°C, and fibrinolysin

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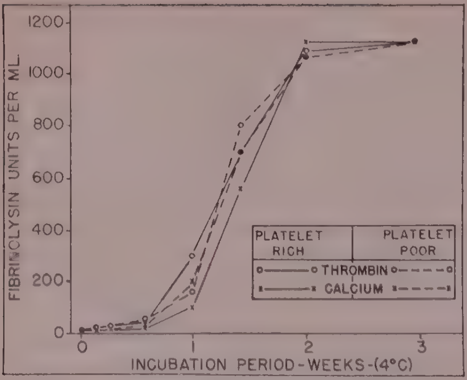

FIG. 1. Appearance of fibrinolysin in chloroformed sera prepared from platelet-rich and platelet-poor plasmas.

contents determined at intervals. In Fig. 1 it is shown that fibrinolytic activity develops at approximately the same rate in each serum regardless of whether the serum has been obtained from platelet-rich or platelet-poor plasma and also regardless of whether the serum is obtained by recalcification or by thrombinization. Control sera, not treated with chloroform, develop no lytic activity.

II. *Does chloroform act as a fibrinolysokinase?* To test whether chloroform itself exerts a kinase-like action on profibrinolysin we used the development of fibrinolytic activity in our standard Prolysin. This serum fraction contains no antifibrinolysin, and does not usually activate spontaneously. The profibrinolysin content of different lots varies between 4000-4600 units per ml.

A solution of Prolysin was divided into 2 lots. One lot was treated with chloroform by shaking with 1/5 volume for 2 minutes. Both lots were incubated at 4°-6°C for 4 weeks and fibrinolysin contents titered twice a week. Neither the plain nor the chloroformed Prolysin developed detectable fibrinolysin titers, although, even after 4 weeks, both still could be shown to contain high profibrinolysin concentrations. Four similar experiments confirmed this result.

III. *"Spontaneous" activation of serum fractions.* Fresh oxalated plasma was recalcified and the resulting serum divided into 2 lots which were fractionated in the following manner. All fractionation procedures were carried out at 5°C. 1. *Euglobulin fraction.* 100 ml serum was diluted to 2000 ml with distilled water and the pH adjusted to 5.35 with 1% acetic acid. After standing cold for one hour, the precipitate was collected by centrifugation and dissolved in 20 ml borate buffer, maintaining pH at 7.6. 2. *Prolysin fraction.* To 100 ml serum was added 49 ml ammonium sulfate (saturated at 5°C). The mixture was allowed to stand for 30 minutes and the precipitate collected by centrifugation. This precipitate was dissolved in distilled water, and dialyzed against running tap water overnight. The resulting precipitate was collected by centrifugation and dissolved in 20 ml borate buffer (pH 7.6). Both fractions were tested for antilysin and profibrinolysin. They were then incubated for 6 weeks at 4°-6°C and fibrinolysin contents titered at intervals. No antifibrinolysin was present in either fraction. Profibrinolysin titers, as determined by staph activation at 5°C were respectively, Euglobulin: 4628 units per ml and Prolysin: 4580 units per ml. As shown in Fig. 2, fibrinolysin appeared spontaneously in the Euglobulin fraction, reaching a titer of 3000 units. The Prolysin fraction did not activate. After the Prolysin fraction had incubated for 6 weeks a sample treated with staphylokinase for 20 minutes at 37°C yielded 1800 units per ml. Similar staphylokinase treatment of the incubated Euglobulin fraction did not yield any increase in fibrinolysin concentration.

From this experiment we concluded that the two fractions, Euglobulin and Prolysin, pre-

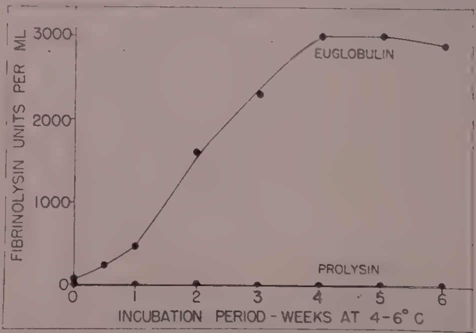

FIG. 2. Appearance of fibrinolysin in Euglobulin and Prolysin fractions of the same serum.

pared from the same serum, were similar in that both were free from antilysin and contained like amounts of profibrinolysin capable of activation by staphylokinase, but they differed in that the Euglobulin activated spontaneously whereas the Prolysin did not. Two explanations seem possible: (1) that the profibrinolysin was so changed in the Prolysin fraction that it could not activate autocatalytically or (2) that an activator, a fibrinolysokinase, was present in the Euglobulin fraction, but absent from the Prolysin fraction.

IV. *Isolation of a fibrinolysokinase fraction from serum.* The last possibility was explored and many attempts were made to isolate from serum a fraction, free from profibrinolysin and antifibrinolysin, but capable of activating Prolysin. Several such fractions, prepared by different methods, were found. The experiment illustrated below describes such a fraction. Pooled whole dog serum was chilled to 5°C and an equal volume of cold absolute ethyl alcohol added. The precipitate so formed was discarded and an additional volume of alcohol equivalent to 24% of the original serum volume was added. The resulting precipitate was collected by centrifugation and dissolved in 1/3 the serum volume of saline. Traces of alcohol were removed by passing a stream of air over the solution. The pH was adjusted to 7.3. Tests of this fraction (K) showed that it contained 82 units of antifibrinolysin (normal whole serum, by this method = 3625 antilysin units per ml) and less than 20 units of profibrinolysin. Mixtures of this fraction (K on Fig. 3) were made with equal volumes of Prolysin and of saline, and a control mixture of Prolysin plus saline was prepared. All pH's were found to be 7.35 ± 0.05. Each mixture was incubated at 5°C and fibrinolysin content determined at intervals.

Fig. 3 illustrates the development of fibrinolysin activity in the various mixtures. After an initial lag the Prolysin plus fraction K mixture (P + K) developed a high titer of fibrinolysin (3730 units) comparable to that developed with this Prolysin plus staphylokinase (4200 units). The Prolysin plus saline (P) developed 49 units of fibrinolysin and the fraction K plus saline (K) developed less than 20 units of fibrinolysin during the 6 weeks incubation period.

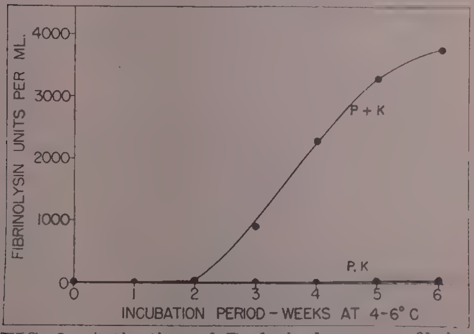

FIG. 3. Activation of Prolysin by serum fibrinolysokinase.

Discussion. The "spontaneous" appearance of fibrinolysin in whole serum which has been treated with chloroform might be explained in any of the following ways: A. The splitting of a fibrinolysin-antifibrinolysin complex with liberation of an active fibrinolysin(6,22). B. A direct action of chloroform on the profibrinolysin molecule. C. The removal of antifibrinolysin followed by the autocatalytic activation of profibrinolysin, as suggested by Christensen and MacLeod(23), or D. The removal of antifibrinolysin (? also antifibrinolysokinase) followed by the activation of profibrinolysin by serum fibrinolysokinase. The first of these possibilities seems unlikely as, immediately after chloroform treatment, antifibrinolysin is greatly reduced or absent, but active fibrinolysin develops only gradually (Exp. I), reaching its maximum in about 2 weeks at 5°C. The second possibility also seems unlikely as chloroform will not activate an isolated prolysin fraction (Exp. II). The third possibility has been explored by attempting to activate our Prolysin by addition of potent fibrinolysin preparations. Activation has been rarely demonstrable and in those cases in which some activation was observed we were unable to eliminate the possibility that the fibrinolysin preparation itself con-

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tained a kinase.

From our first experiment and from previous experiments(3) in which no kinase activity could be found in isolated platelets, leukocytes or erythrocytes, we concluded that if a fibrinolysokinase was responsible for the "spontaneous" activation of profibrinolysin it must be a serum component independent of cellular elements.

Experiment III strongly suggests that the Euglobulin fraction of serum contains a fibrinolysokinase which is absent in the Prolysin fraction. The isolation and action of such a substance is shown in Experiment IV. Fraction K was able to activate the Prolysin almost completely in the 6 week experimental period. The slow initiation of this activation may be due to the presence of traces of antifibrinolysin in Fraction K. Other serum fractions, prepared from whole serum and from the serum residual after Prolysin has been removed, have also proved active as fibrinolysokinases. Apparently very exact technic is necessary to separate the kinase from profibrinolysin as duplicate experiments on different serum lots will not always give a good separation. Studies are underway to elucidate further this fractionation problem.

Conclusion. Serum, independent of cellular elements, contains a fibrinolysokinase which is capable of converting profibrinolysin to active fibrinolysin.

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Relation of Vitamin E to Acute Physiological Stress.* (19015)

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According to Selye(1), the normal physiological response of the body to varied forms of stress, such as cold, heat, emotional excitement, etc., proceeds via a common pathway. The pathway seems to involve stimulation of the pituitary gland by epinephrine or other agents. The pituitary then releases adrenocorticotropic hormone (ACTH), which causes the adrenal glands to secrete various steroid hormones (*e.g.* cortisone) which mediate the physiological response of the body to stress. The entire system is designated the "general adaptation syndrome."

The nutritional status of the body has been shown to affect the capacity to respond to stress in the case of several nutrients such as pantothenic acid(2) and vit. C(3). The possibility of a causal relationship between vit. E and the stress response via the anterior pituitary-adrenal cortical pathway seemed likely in view of the known protective effect of α-tocopherol in various different types of stress, such as carbon tetrachloride poisoning (4), exposure to high altitude(5), or dietary restriction of either protein or total calories (6). Also, vit. E has been implicated as a factor in maintaining the anatomical integrity of the adrenal cortex by Tonutti(7) who found that vit. E-deficient rats showed degenerative changes in the exterior and interior zones of the adrenal cortex similar to those following hypophysectomy.

Two experiments were performed to test the possibility that vit. E is related to the initial phase (alarm reaction) of the general adaptation syndrome.

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TABLE I. Effect of Acute Stress on Adrenal Ascorbic Acid Content of α-Tocopherol-Supplemented and of Vit. E-Deficient Rats.

	Ascorbic acid in adrenals (mg/100 g)			
	No stress		Stress	
	(—E) rats	(+E) rats	(—E) rats	(+E) rats
Mean values (5 exp.)	396	427	254	276
S.E.	± 27	± 57	± 27	± 33

Mean % decrease due to stress:

$$\text{for } (+\text{E}) \text{ rats} = \frac{427\text{–}276}{427} \times 100 = 35.2\%$$

$$\text{for } (—\text{E}) \text{ rats} = \frac{396\text{–}254}{396} \times 100 = 35.9\%$$

Exp. I. Effect of vit. E nutriture on response of the rat to acute stress. Adrenal ascorbic acid content. The change in concentration of ascorbic acid in the adrenal gland is a well-known criterion of response to stress via the pituitary-adrenocortical system(8). Hence, the concentration of ascorbic acid in the adrenal glands of both stressed and unstressed rats of groups which were markedly E-deficient and of those which were identical except for continuous α-tocopherol supplementation was measured. Thirty male weanling rats of Holtzman stock were maintained on an otherwise adequate, vit. E-deficient diet No. 301(9) for 2 months, during which time one-half received d-α-tocopherol supplements, totaling 12 mg/week, by oral administration. A series of "stress" experiments was then performed on 4 rats at a time, 2 from each group, all of approximately equal body weight. Animals were used after a 6-hour fast. A tocopherol-supplemented and a tocopherol-deficient rat were each subjected to stress, while a tocopherol-supplemented and a tocopherol-deficient rat were not, as controls. "Stress" consisted of subcutaneous injection of 0.1 mg of epinephrine-HCl in 0.1 ml of distilled water in every experiment but one. In this experiment "stress" consisted of exposure to cold (5°C) for 1 hour. Controls received an injection of distilled water or in the one experiment were maintained at room temperature. The animals were killed 1 hour after injection by a blow on the head. The content of ascorbic acid in the adrenal glands of each rat was immediately determined by the method of Mindlin and Butler(10) on a 2.5% metaphosphoric acid extract of the glands made with a glass (Potter-Elvehjem) homogenizer.

Results are given in Table I. The mean decrease of ascorbic acid concentration of tocopherol-supplemented rats in response to stress was 35.2% which approximates values for normal rats, reported in the literature. The corresponding change for tocopherol-deficient rats was a decrease of 35.9%.

Thus, no difference in amount of pituitary-induced adrenal cortical activity as a result of acute stress was found between tocopherol-supplemented and tocopherol-deficient rats.

Blood concentrations of vit. A and E. Blood sera of rats in some of the experiments were analyzed for their content of vit. A and E by the micro methods of Lowry *et al.*(11,12). Results are shown in Tables II and III.

Tocopherol-deficient rats, with or without stress, had zero blood tocopherol levels. The supplemented groups had levels of 1.72 mg % (stress) and 1.68 mg % (no stress). The contrasting nutritional status of tocopherol-supplemented and unsupplemented rats with respect to vit. E is thus amply confirmed. Also is shown the lack of effect of stress on the

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TABLE II. Effect of Acute Stress on Blood Serum Vit. E Levels.

	Total tocopherols in serum (mg/100 ml)			
	Stress		No stress	
	(+E) rats	(—E) rats	(+E) rats	(—E) rats
Mean values (4 exp.)	1.72	0	1.68	0
S.E.	± .11	0	± .06	0
% change due to stress:				
for (+E) rats $= \frac{1.72-1.68}{1.68} \times 100 = +2.4\%$				
for (—E) rats = 0%				

TABLE III. Blood Serum Vit. A Levels of Rats Subjected to Acute Stress.

	Blood serum Vit. A (μg/100 ml)			
	Stress		No stress	
	(+E) rats	(—E) rats	(+E) rats	(—E) rats
Mean values (3 exp.)	44	31	46	41
S.E.	± 1	± 4	± 6	± 2
Mean % change due to stress:				
for (+E) rats $= \frac{44-46}{46} \times 100 = -4\%$				
for (—E) rats $= \frac{31-41}{41} \times 100 = -24\%$				

blood tocopherol levels of the animals.

Mean blood vit. A levels were essentially equal for 3 of the 4 groups and markedly lower for the one "stressed", unsupplemented group. The mean changes of blood vit. A levels following stress, were –4% for the tocopherol-supplemented rats and –24% for the tocopherol-deficient rats. These data are too few for conclusive results; however, they suggest that vit. E may have "protected" vit. A in the blood during stress. These blood vit. A values fail to confirm the report of Young and Wald(13) that epinephrine injection caused a pronounced increase in the blood content of vit. A. They agree with results of Goodwin and Wilson(14). Both groups used rabbits in their experiments.

Exp. II. Effect of acute physiological stress on the tocopherol content of the adrenal glands of normal rats. Mature, well-nourished male rats were used as experimental subjects. Fifteen litter-mate pairs of weanling male rats from the Distillation Products Industries' stock colony were maintained on Diet 1, complete stock breeding diet, until they reached about 400 g weight (about 5 months). One pair was used as a check on the efficacy of the particular stress used here in eliciting the pituitary-induced, adrenal-cortical response. After overnight fast, one rat received 0.1 ml of epinephrine-HCl (aq.) (1 mg/ml) by subcutaneous injection, while the control received distilled water by the same route. They were killed after one hour, by a blow on the head, and the adrenal ascorbic acid concentration was measured. The stressed rat had 213 mg/100 g of ascorbic acid in his adrenal glands and the control animal 336 mg/100 g. The decrease caused by stress, *i.e.* 36.6% of the control, was nearly identical to the mean value of a series of rats (both vit. E-deficient and sufficient) tested previously by the same technic (Exp. I). Thus, the method of physiological stress used did induce the expected pituitary-adrenal response in these rats. The remaining 14 pairs of litter-mate rats were stressed similarly, 1 or 2 pairs at a time. After overnight fast, they were injected with either 0.1 mg of epinephrine (stressed rats) or 0.1 ml of distilled water (controls). After one

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TABLE IV. Effect of Stress Upon Vit. E Content of the Adrenal Glands of Normal Rats.*

Group	Mean adrenal wt (mg)	Mean body wt (g)	Total tocopherol in the adrenals	
			Mean for group (mg/100 g of adrenals)	Mean of differences between litter pairs (mg/100 g of adrenals)
No stress	39.78	392	30.8	3.7 ± 4 (S.E.)†
Stress	38.64	380	34.6	

* One of each of 14 litter-mate pairs of male rats, raised on a complete stock breeding diet, was subjected to physiological stress (0.1 mg epinephrine by subcutaneous injection) 1 hr before sacrifice.

† This difference is not statistically significant ($p = 0.4\text{-}0.3$) by the Fisher "t" test.

hour, the animals were killed by a blow on the head and the adrenals removed and carefully dissected free of fat. They were weighed immediately to the nearest 0.01 mg on a Roller-Smith balance. Each pair of adrenals was then analyzed for content of total tocopherols. The extracts for analysis were prepared by homogenizing pairs of adrenals in all-glass apparatus (Potter-Elvehjem) in 6 ml of 1:1 ethanol-xylene, adding 3 ml of H_2O, agitating, and centrifuging. Aliquots of the xylene layer were then analyzed spectrophotometrically as in the micro method of Quaife *et al.*(12).

Results. Experimental data, including mean body weight of rats, mean adrenal weight, mean vit. E concentration in the adrenals (mg total tocopherols/100 g of adrenals) are summarized in Table IV. Also the mean of differences in adrenal tocopherol content for each pair (stressed vs. control) is given.

The apparent slight increase in tocopherol content of the adrenal glands, due to stress, was shown to be without statistical significance ($p = 0.4\text{-}0.3$).

Summary and conclusions. The nutritional status of rats with respect to vit. E had no effect on the adrenal cortical activity (as measured by change in adrenal ascorbic acid concentration) following acute physiological stress (epinephrine injection or exposure to cold). Neither the content of vit. E in the adrenal glands of normal adult rats nor the blood tocopherol content of tocopherol-supplemented or tocopherol-deficient rats was changed following physiological stress.

Thus, vit. E is apparently not a factor in the anterior pituitary-adrenal cortical response to acute physiological stress, although vit. E may be related to the manner in which the tissues utilize the adrenal cortical hormones released as a result of such stress.

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Chloromycetin Palmitate.* Its Use in Pediatrics. (19016)

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Chloromycetin is often difficult to administer to infants and children because of its bitter taste. Esters of Chloromycetin with higher fatty acids have been prepared in an attempt to produce a palatable form of the drug. One of these esters, Chloromycetin Palmitate, is now under clinical investigation. The ester is formed by replacing one of the hydroxyl groups of the propane diol side chain of Chloromycetin with Palmitic acid. Chloromycetin Palmitate has not previously been used in clinical medicine. Animal experi-

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* The author is indebted to Parke-Davis and Co. which supplied the Chloromycetin Palmitate suspension and which gave permission to quote their studies of toxicity of the drug in animals.

TABLE I. Infections Treated with Chloromycetin Palmitate–Bacteriological Studies.

Case No.	Age	Diagnosis	Organism	Source	*In vitro* sensitivity to chloromycetin	Result of treatment
1	9 yr	Chronic pyuria	*E. coli**	Urine	Yes	Negative culture
2	2	Acute pharyngitis	Paracolobractrum	Throat	None	Symptoms subsided
3	2	Acute purulent otitis media	Streptococcus *Staphylococcus aureus*	Ear "	Yes "	Ear normal
4	9	Vaginal discharge, 2 wk	Pneumococcus	Vagina	Not done	Discharge subsided promptly
5	21 days	Impetigo neonatorum, 2 wk	*Staphylococcus areus*†	Skin	Yes	Healed promptly
6	12	Diarrhea, 5 days	*Salmonella typhimurium**‡	Stool and Blood	" " "	Cultures of stool Blood neg
7	3 mo	Cystic fibrosis of pancreas	*Staphylococcus aureus*	Throat	"	Improved
8	2	Cystic fibrosis of pancreas	"	Duodenum and throat	"	"

* Not responsive to aureomycin. † Resistant to penicillin. ‡ Treated also with terramycin.

TABLE II. Common Infections of Children Treated with Chloromycetin Palmitate.

Case No.	Age	Diagnosis	Result of treatment
9	9 mo	Acute bronchitis	PR‡
10	2 yr	" gastroenteritis	"
11	18 mo	" "	"
12	11 days	Impetigo neonatorum	"
13	4 yr	Multiple furunculosis	"
14	7	Acute follicular tonsillitis	"
15	5	Bronchopneumonia* RLL, RML, LLL	"
16	2	Acute cervical adenitis	"
17	4	Pertussis†	CRI§

* Penicillin and sulfonamide resistant. † Not responsive to Terramycin. ‡ PR = prompt recovery. § CRI = cough rapidly improved.

ments, however, indicate that it is less toxic than Crystalline Chloromycetin.

A suspension of Chloromycetin Palmitate for pediatric use, containing 125 mg of Chloromycetin per 4 cc (one teaspoonful) was used in the treatment of 17 patients with a variety of diseases. Ages varied from 11 days to 9 years. Dosage was 50 mg per kg of body weight in 24 hours, divided in 4 or 6 equal doses and continued until the patient was clinically cured, from 3 to 7 days.

Table I shows 8 patients in whom bacteriological studies and *in vitro* antibiotic sensitivity tests were carried out. All patients in this group responded promptly to treatment with Chloromycetin Palmitate.

Table II shows 9 patients who received Chloromycetin Palmitate for conditions which are commonly encountered in children. No bacteriological studies were carried out in this group. All patients responded promptly to therapy.

Parents of these 17 patients reported that the Chloromycetin Palmitate suspension was taken without difficulty and in most cases with pleasure. No instances of nausea, vomiting, diarrhea, drug fever or drug eruption was noted in this small series.

Summary. A new preparation of Chloromycetin, in the form of Chloromycetin Palmitate suspension, was administered to 17 patients ranging from 11 days to 9 years of age.

The preparation is palatable and taken readily by these patients. A variety of mild and serious common childhood illnesses responded promptly to this medication.

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Histology of the Brain and Blood in Chronic Experimental Sedation with Barbiturates and Presidon.* (19017)

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The appearance of histologic changes in the central nervous system following indiscriminate use of barbiturates is held as a major argument against the unrestricted and prolonged use of these drugs. However, the evidence for this idea is not strong, especially in human beings. Hassin's(1) report on acute intoxication in man and Neumann's(2) recent report of a case surviving severe intoxication for five months show only non-specific changes in the brain that could well be the result of anoxia due to respiratory depression by the drug. More conclusive evidence as to effects of barbiturates on the human brain is lacking. Seevers and Tatum(3) examined dogs maintained for as long as three years on daily barbital administration. The post mortem examination showed thickened meninges and walls of cerebral vessels with some degeneration of neurons of the brain. However, such damage was poorly localized, varying in degree and location with the individual animals. Krop and Gold(4) could find no changes in the brain of the dog following barbiturate intoxication with lethal dosage. The present study was undertaken to compare the effects of barbiturates with those of 3,3 diethyl-2,4 dioxotetrahydropyridine, Presidon, a non-barbiturate hypnotic, on the histology of the central nervous system of dogs chronically sedated over long periods of time.

Method. Twelve mongrel dogs, fed on a stock diet throughout the experiment, were divided into two groups of six. Three animals of each group were given a minimum hypnotic dose of Presidon twice daily, two animals received a similar dose of barbital, and one animal received phenobarbital. The animals in one group were sacrificed after six months, and those in the remaining group after one year. The animals were sacrificed during the sleep following their daily drug intake, the brains perfused with formalin (U.S.P. 1:10), and the viscera examined grossly and samples taken for microscopic examination. The spinal cords and brain stems were frozen by Marshall's technic and sectioned serially. The cerebral and cerebellar cortices were sampled and prepared for examination in a similar manner. Alternate sections were stained with thionin, Weil's and a few with Masson's stains. During the last seven months of the experiment complete blood counts were made biweekly on all the dogs, and a sample of sternal marrow was taken at autopsy in order to observe any possible development of blood dyscrasias especially agranulocytosis which has been reported clinically following the use of Presidon.

Results. A single dose of Presidon produced a light sleep of about two hours duration. The dogs could always be aroused from such sleep at any time and upon undisturbed awakening were immediately alert. In none of the animals receiving this drug were overt

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* This work was supported by a grant from Hoffmann-LaRoche, Inc., to whom we are also grateful for supplies of the drugs used.

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TABLE I. Drug Dosages and Blood Counts of Treated Dogs.

	Daily oral dosage*		Blood counts			
Dog No.	Drug	Daily dose (mg/kg)	No. of counts	Initial WBC	Avg WBC	Avg RBC (million/cmm)
1	Barbital	106	13	16550	11104	7.43
2		121.5	13	15350	12893	8.25
3		103	2	12500	10375	6.55
4		152	2	12400	13400	6.13
5	Phenobarbital	61.3	13	10450	14400	6.25
13		70.8	2	12300	10800	5.25
7	Presidon	342	2	8150	10500	4.65
8		333	2	18750	13675	5.86
9		353	2	11050	11750	7.44
10		312	13	7600	10290	8.20
11		343	13	8400	12704	7.53
12		361	13	8300	9139	7.27

* Range of body wt—7.3 to 15.8 kg.

neurological disturbances observed even after 12 months of continuous medication. Barbital and phenobarbital, on the other hand, resulted in periodic sleeping which was inconstant both in time of appearance and duration; furthermore, there was a tendency for accumulation of the effect of these drugs so that often the animals would be deeply narcotized for hours necessitating the omitting of the drug for a day. Awakening from sleep in these dogs was accompanied by ataxia and general disorientation for several minutes, and the animals receiving barbital for one year were constantly mildly ataxic during the last two months of the experiment. Withholding the drugs for as long as 4 days never resulted in convulsions in these dogs, although the animals on barbital and phenobarbital became quite restless.

Necropsy findings upon the gross and microscopic examination of the viscera were in general devoid of significant changes. The spleens of the animals receiving barbital and phenobarbital were the exception. Pinkish gray tumors, 2 to 3 mm in diameter, lined the border of the spleen, and microscopic examination showed them to contain splenic tissue and large vessels congested with erythrocytes. There were no histologic changes in the central nervous system of any of these dogs that could be classified as pathologic in the usual sense. Perhaps an exception to this was the presence of small round elements similar to the amyloid bodies often seen in the human brain including presumably normal cases. These were scattered through the brain stem and cortex. Mott, Woodhouse, and Pickworth(5) described similar stuctures in the brains of cats and monkeys chronically intoxicated with barbiturates. However, their chemical analysis showed such bodies to be mucinoid, rather than amyloid in composition, and they presumed them to be of degenerative origin. A chemical analysis was not made in our experiment, and it is perhaps possible that these structures may be indicative of some change in brain chemistry since they appeared only in the animals receiving the drug for one year.

Table I lists the results of the blood counts of these animals, and the values are compatible with those for normal dogs as given by Mayerson(6) and Wells and Sutton(7). The post mortem examination of the sternal marrow showed ample amounts of all myeloid leucocytic elements, and the peripheral blood was free of immature leucocytes.

Discussion. The failure to observe histologic changes aside from the amyloid-like bodies in these animals seems somewhat in disagreement with the literature; however, when considering the differences in dosage, condition of the animals and method of preserving the brain, a latitude in results of different experiments is most likely, and as has

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been mentioned no very marked changes in the brain of the dog have been previously observed following chronic barbiturism. Lack of histologic change, however, does not necessarily preclude the possibility of a derangement of physiological processes, and the ataxia of the barbital dogs is a good indication of this fact. Failure to observe further neurological signs or withdrawal symptoms may very well be due to the dosages used which, as can be seen in Table I, were not large when compared to the daily intake in human barbiturate addiction. Table I also shows that large doses of Presidon were needed to produce sleep as compared to small doses of the barbiturates, yet the sleep after the former was much more uniform without cumulative symptoms or after effects such as ataxia or disorientation. The presence of the amyloid-like bodies could not in this experiment be related to any neurological signs for they were present in the dogs receiving Presidon as well. However, their appearance only in those animals treated for one year does suggest a relationship to prolonged use of these drugs.

Whether or not there are species differences in reactions to these drugs is of course not determined. However, in comparing our results with the literature, it would seem such differences, at least with regard to the nervous system, would be a matter of degree. With regard to the blood, on the other hand, species differences may be a factor in the development of dyscrasias following the use of drugs. The dog appears to have a much more active hemapoietic system than man and conceivably could be more resistant to drug effects.

Summary. (1) Twelve dogs receiving barbital, phenobarbital or Presidon daily for six months to one year were observed for neurological disturbances and alterations in the histology of the viscera, blood and brain. (2) Dogs receiving barbital for one year developed a mild ataxia. These dogs and one receiving phenobarbital for one year had small splenic tumors at autopsy. All other post mortem findings were negative with respect to histologic changes in the viscera and blood. Small structures similar to amyloid bodies seen in man were found in the brain stem and cortex of all dogs treated one year, but not in dogs treated six months. These structures could not be correlated with neurological changes in behavior, nor is their significance known.

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Mechanism of Enzymatic Reduction of Triphenyl Tetrazolium Chloride.* (19018)

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Although considerable attention is being paid to tetrazolium salts in various fields of biology(1,2) the mechanism of their reduction by mammalian tissues requires further elucidation. It can be assumed that in an enzymatic reducing system, composed of an enzyme protein, pyridine-nucleotide and flavoprotein, the immediate electron donor of triphenyl tetrazolium chloride (TTC) is the dye-reducing flavo-protein of Straub(3). This mechanism was suggested by Bieling, *et al.*(4) and Kun(1) and clearly demonstrated by Brodie and Gots(5) with yeast triose phos-

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† Present address: Tulane University of La., Departments of Medicine and Biochemistry.

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phate dehydrogenase. The present paper deals with the mechanism of the intracellular enzymatic reduction of TTC by rat tissues.

Materials and methods. Enzymes. The dialyzed cytoplasmic centrifuged fraction of tissue homogenates (in 0.15 *M* KCl, containing ⅕ volume of 0.1 *M* phosphate buffer of pH 7.4) served as a source of soluble dehydrogenases. Amino acid dehydrogenases(6-8) were extracted from rat kidney cortex, glycolytic enzymes (enzymes of type A) from rat liver or muscle. Mitochondria were prepared from rat liver homogenates according to Schneider and Hogeboom(9) and extracts of mitochondria by the isobutanol procedure, as described previously(10), except that the extraction was carried out at 37°C for 45 minutes (Enzyme B). The procedure of Racker was employed for the preparation of yeast alcohol dehydrogenase(11). *The potassium salt of fructose-1-6-diphosphate* (HDP) was prepared from a commercial sample of the calcium salt (Schwarz). The inhibitory effect of eventually occurring heavy metal contaminants could be abolished by the use of histidine hydrochloride, in a final concentration of 10^{-2} *M*(12). The TTC was a purified sample, generously given by Dr. George Gomori. *Enzyme activity* was determined spectrophotometrically as described previously (13). The enzyme activity is expressed in terms of optical density x 10^3 of the acetone filtrate as measured at 484 mμ on the Beckman spectrophotometer in 1 cm Corex cells (3 ml content). Distilled water served as a blank.

TABLE I.

	TTC reduction	Cytochrome C reduction
Mitochondria	+ (100)	+ (100)
Cold (0°C) isobutanol ext.	—	+ (40–70)
Warm (37°C) ”	+ (40–60)	

Results. Typical flavoproteins, like amino-acid dehydrogenases(6-8), present in kidney extracts, directly reduce TTC. The rate of reduction in the presence of l-alanine or d-aspartic acids is 6-12 times greater under anaerobic conditions, than in air. Unlike aminoacid dehydrogenases, triose phosphate dehydrogenase, present in liver or muscle extracts (Enzyme A) does not react with TTC either aerobically or in N_2 atmosphere. Mitochondria prepared by the sucrose procedure(9) do not reduce TTC with HDP as substrate. Rapid reduction of TTC occurs, however, when mitochondria are added to the glycolytic enzyme systems (Enzyme A). With an excess of glycolytic enzymes present, the rate of TTC reduction is a function of the amount of mitochondrion particles. It is apparent that the reduction of TTC by triose phosphate dehydrogenase (present in Enzyme A) occurs by way of mitochondria. Cytochrome C reductase activity is also known to be bound to mitochondrion particles(14). It was therefore of interest to inquire whether or not the TTC and cytochrome C reducing systems have a catalyst in common. It was previously reported that the cytochrome reductase system of mitochondria could be extracted by treatment with isobutanol at 0°C (10). This cytochrome C reductase system did not reduce TTC. On the other hand, when the extraction of mitochondria was carried out at 37°C, TTC reductase was obtained, and cytochrome C reductase activity lost. The catalytic properties of the mitochondrion extracts prepared at 0°C and at 37°C can be compared, by taking the activity of intact mitochondria as 100 (Table I).

The role of mitochondria and that of the warm isobutanol extract of mitochondria (Enzyme B) in TTC reduction is summarized in Table II, which represents typical experi-

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TABLE II. The Glycolytic Reduction of TTC.

Enzyme	TTC reduction in terms of optical density × 10^3	Incubation time
Rat muscle ext. (Enzyme A type) (1)	0	30′ aerobic
Rat liver mitochondria (2)	46	30′ ”
Muscle ext. (1) + mitochondria (2)	500	15′ ”
Rat liver ext. (Enzyme A type) (3)	20	15′ anaerobic
Mitochondrion ext. (37°C) (Enzyme B) (4)	0	15′ ”
Rat liver ext. (3) + mitochondrion ext. (4)	720	15′ ”

Enzyme preparations: (1) = muscle ext. equivalent to 0.5 g fresh rat muscle (Enzyme A). (2) = rat liver mitochondria equivalent to 0.140 g fresh liver. (3) = rat liver ext. equivalent to 0.2 g fresh liver (Enzyme A). (4) = mitochondrion ext. equivalent to 0.3 g fresh liver (Enzyme B).

The glycolytic system is composed of: 60 μM HDP, 40 μM nicotinamide, 40 μM KF, 0.6 μM DPN and 3 μM TTC in a final volume of 3 ml made up with 0.1 M phosphate buffer. Temp. 37°C.

mental results. Essentially the same results were obtained when the triose phosphate dehydrogenase system of tissue extracts was replaced by crystalline yeast alcohol dehydrogenase(11). The reduction of TTC by the alcohol dehydrogenase system was measured anaerobically at pH 7.4, with semicarbazide hydrochloride (10^{-2} M) as carbonyl reagent. Here again, TTC reduction occurred only when mitochondria, or the extract of mitochondria (Enzyme B) were present in addition to the yeast enzyme, Table II.

Discussion. In animal tissues TTC can be reduced by a variety of dehydrogenases. Soluble flavoproteins, like aminoacid dehydrogenases, readily transfer electrons to TTC under anaerobic conditions. The retardation of TTC reduction by aminoacid dehydrogenases in air is probably due to a competitive mechanism between O_2 and TTC, particularly noticeable in homogenous systems (solutions). It was shown that an obligatory component of the glycolytic TTC-reducing system is present in mitochondria. This component appears to be a flavoprotein part of the cytochrome C reducing system(10). The fact that intact mitochondria mediate electrons to both cytochrome C and TTC, while the two reductase activities can be separated by extraction, requires further elucidation.

Summary. The conditions of the reduction of TTC by cytoplasmic fractions of rat tissue homogenates were determined. Rat kidney extracts, upon addition of aminoacids, reduce TTC anaerobically. Glycolytic enzymes of liver and muscle reduce TTC only when supplemented by mitochondria. An extract of mitochondria can be obtained which will supplement the glycolytic TTC reducing system.

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Retardation of Growth of Walker Rat Carcinoma 256 by Administration of Diethyl Riboflavin.* (19019)

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In recent years the effects of riboflavin deficiency on the growth of neoplasms have received increasing attention. Morris and Robertson(1) found that the growth rate of spontaneous mammary carcinomas in C3H mice was decreased by severe riboflavin de-

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ficiency. Stoerk and Emerson(2) have reported the regression of established lymphosarcomas (6C3H-ED) in mice rendered riboflavin deficient by either feeding a flavin deficient diet or by administering the riboflavin antagonists, isoriboflavin or galactoflavin. Another riboflavin analogue, 6,7-dichloro-9-(1′-D-sorbityl)-isoalloxazine, which has no antiriboflavin activity in rats, also causes regression of established lymphosarcomas(3).

Lambooy(4) has recently reported the synthesis of 6,7-diethyl-9-(D-1′-ribityl)-isoalloxazine. Lambooy and Aposhian(5) have shown that this new flavin has an "antiriboflavin" activity in the growing rat. The effects of this new flavin on the Walker Rat Carcinoma 256 are reported in this paper.

Methods. Twenty-four male rats of the Sprague-Dawley strain, weighing from 31 to 45 g were fed a riboflavin deficient diet consisting of sucrose 68%, vitamin test casein 18%, vegetable oil (hydrogenated) 10%, and salt mixture No. 2 USP 4%. Each 100 g of diet was supplemented† with 2 g USP cod liver oil, 5 mg each of vit. K_1 and nicotinamide, 1 mg each of thiamine and pyridoxine, 2.5 mg calcium pantothenate, 10 mg 1-inositol, 100 mg choline chloride, 60 μg each of folic acid and biotin, 3 μg B_{12} and 30 mg para-aminobenzoic acid. A drop of mixed tocopherols containing 3 mg α-tocopherol per drop was administered orally to each animal once a week. On the thirteenth day the animals were paired into two groups according to weight and previous rate of growth and slice fragments‡ of the Walker Rat Carcinoma 256 were implanted aseptically by trocar into the subcutaneous tissue of the right inguinal region of the rats. Group A received 32.3 μg

TABLE I. Comparison of the Effects of Diethyl Riboflavin and Riboflavin on Walker Carcinoma 256.

Diethyl riboflavin Group A						Riboflavin Group B							
Final wt* (r + t)	Tumor wt* (t)	Carcass wt* (r—t)	Initial wt (i)	Net change (r—t—i)	Tumor size ($\frac{t}{r+t}$ x 100)	Final wt* (r + t)	Tumor wt* (t)	Carcass wt* (r—t)	Initial wt (i)	Net change (r—t—i)	Tumor size ($\frac{t}{r+t}$ x 100)	Δ Net change	Δ Tumor size
g	g	g	g	g	%	g	g	g	g	g	%	g	%
88	26	62	72	—10	30	98	26	72	79	— 7	27	— 3	— 3
90	11	79	76	+ 3	12	107	29	78	83	— 5	27	+ 8	+15
105	9	96	81	+15	9	120	32	88	81	+ 7	27	+ 8	+18
75	22	53	59	— 6	29	71	23	48	74	—26	32	+20	+ 3
100	20	80	70	+10	20	116	41	75	72	+ 3	35	+ 7	+15
90	12	78	73	+ 5	13	106	34	72	77	— 5	32	+10	+19
78	7	71	66	+ 5	9	70	15	55	65	—10	21	+15	+12
77	20	57	69	— 12	26	84	25	59	69	—10	30	— 2	+ 4
69	13	56	65	— 9	19	75	25	50	69	— 19	33	+10	+14
78	12	66	66	0	15	90	29	61	74	—13	32	+13	+17
81	21	60	72	—12	26	88	23	65	74	— 9	26	— 3	0
74	23	51	60	— 9	31	73	24	49	71	—22	33	+13	+ 2
				Mean	20						30	+ 8	+10

* At time of sacrifice.

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† We are indebted to Merck and Co. for the generous gift of the vit. K_1 and B_{12} used in this study.

diethyl riboflavin§ and Group B received 30 μg riboflavin daily beginning with the day of tumor implantation. The flavins were administered by stomach tube as a gum acacia suspension.

Animals of Group A were allowed to eat freely. The food intake of each Group B rat was restricted to the food intake of its Group A pair mate. The weight of the animal on the day that the tumor was first palpable was designated as its initial weight. At the first sign of ulceration in any rat (no significant amount of fluid was lost) the pair of animals was decapitated, their tumors removed and weighed.

Results. See Table I. Net change (carcass weight—initial weight) is an expression of the change in weight of the animal carcass (rat weight—tumor weight) from the time the tumor was first palpable to the time of sacrifice. A positive net change indicates that while there was a tumor growing in the host, the animal carcass was gaining weight. A negative net change indicates the reverse.

By first calculating the net change of each animal, then finding the difference between the net changes of the two animals comprising each pair (Δ net change) and computing the average Δ net change of twelve pairs, the twelve rats receiving diethyl riboflavin had a highly significant average net change of 8 g more positive than the rats receiving riboflavin (S.E. = ± 2 g; p = 0.002).¶ This means that the diethyl riboflavin animals were able to retain 8 g more of their carcass tissue than the riboflavin animals.

By calculating first the size of the tumors of each animal expressed as per cent of total body weight ($\frac{t}{r+t} \times 100$), then finding the difference between the size of the tumors of the two animals comprising each pair (Δ tumor size) and finally finding the average Δ tumor size of 12 pairs, it is concluded that the diethyl riboflavin group had tumors whose size, expressed as per cent of total body weight, averaged 10% less than the group of rats receiving riboflavin. This difference in tumor size is highly significant (S. E. = ± 2%; p = 0.001). However, if the average tumor size (20%) of the diethyl riboflavin group is compared to the average tumor size (30%) of the riboflavin group, it is seen that diethyl riboflavin caused a 33% inhibition in the growth of the tumor.

Although no daily quantitative measurements were made, the growth rate of the tumors of the riboflavin animals were strikingly greater than that of the diethyl riboflavin animals. That these changes have been brought about by the administration of unusually small amounts of the riboflavin analogue seems worthy of emphasis. The smallest amount of riboflavin analogue previously used in tumor studies has been 0.5 mg in mice(2).

The average time between implantation of these tumors and the sacrificing of the animals was 21 days. The time of sacrifice of a pair was determined by the ulceration of the tumor in the riboflavin animal in 11 of the 12 pairs.

Studies are now in progress to elucidate the mechanisms by which diethyl riboflavin is able to retard the growth of the Walker Rat Carcinoma 256.

Summary. 1. Rats with Walker Carcinoma 256 transplants showed an 8 g greater retention of their carcass tissue when receiving diethyl riboflavin than pair fed controls receiving an equimolar amount of riboflavin. 2. The average tumor size, expressed as per cent total body weight, of the diethyl riboflavin group was 20% while the average tumor size of the riboflavin group was 30%. This indicates a 33% inhibition of the growth of the tumors due to the administration of diethyl riboflavin.

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§ Equimolar with 30 μg riboflavin.

¶ Standard error; probability.

Radioactive Eggs. II. Distribution of Radioactivity in the Yolks. (19020)

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The distribution of radioactivity in the yolks of eggs laid by a hed fed sodium acetate-1-C^{14} has been determined. The method of feeding and the gross distribution of radioactivity in the shell, yolk and albumen has already been described(1). The 10 yolks discussed below include 7 eggs laid during the period of feeding, 2 large ova taken from the hen (a Single Comb White Leghorn) after sacrifice and the last fraction consists of the combined small ova.

The dried yolks were separated into protein, phospholipid, fatty acid, cholesterol, and glycerol fractions. In each case, activity was present in every fraction. The rapidity of incorporation of radioactivity is demonstrated by the finding that all fractions of the first yolk, laid within 24 hours after feeding was begun, contained measurable amounts of activity. The fact that this yolk was, presumably, completely formed about the time that feeding was initiated(2) further accentuates the speed with which acetate is incorporated into all fractions. In every case the specific activity of the cholesterol was higher than that of any other fraction. Generally, the order of specific activity of the other fractions was fatty acids, phospholipid, glycerol and protein, although in the second egg the glycerol specific activity was second to that of cholesterol. Only the specific activity of the glycerol was the same order of magnitude throughout, the specific activity of all other fractions increased with each succeeding egg. The peak activity was reached in the sixth egg in the case of protein and glycerol; the seventh in the case of cholesterol and the fatty acid activity was at the same high level in the fifth and sixth eggs. The high specific activity of the cholesterol may be explained in either of two ways. Either the acetate is directly incorporated into this component, which view might be supported by the higher specific activity of this fraction in the first egg and also that the peak activity of the cholesterol occurs in the seventh yolk and not in the fifth or sixth, as with other fractions. The seventh egg was laid after all feed had been exhausted and it could be argued that the acetate was already incorporated into cholesterol, whereas conversion to the other components had not yet occurred. On the other hand, there is evidence(3-5) that fatty acids can be converted to cholesterol and the theory that fatty acids, once formed, are further metabolized to cholesterol could also explain the higher specific activity of this component.

In a previous publication(1) the abnormally low value obtained for the specific activity of yolk 5 was ascribed as probably being due to an error in combustion. The present results seem to confirm this conclusion, namely, that the original low value was the result of an error in technic rather than being due to any abnormality in egg production.

Experimental. Protein. The dried egg yolks were pulverized and extracted with ether-alcohol 1:3 in a Soxhlet apparatus for 100 hours. Extraction for longer periods caused no weight loss in the protein residue. The dried residue was converted to carbon dioxide by the Van Slyke wet combustion procedure (6). The carbon dioxide was collected as

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TABLE I. Specific Activities of Yolk Fractions ($\mu c/mg$).

Yolk #	Protein	Phospholipid	Cholesterol	Fatty acids	Glycerol
1	2.85×10^{-5}	1.74×10^{-4}	3.51×10^{-3}	7.77×10^{-4}	3×10^{-3}
2	4.38×10^{-4}	1.17×10^{-3}	3.87×10^{-3}	3×10^{-3}	6×10^{-4}
3	1.13×10^{-3}	3.44×10^{-3}	7.86×10^{-3}	7.86×10^{-3}	1.90×10^{-3}
4	1.67×10^{-3}	6.16×10^{-3}	2.99×10^{-2}	1.47×10^{-2}	3×10^{-3}
5	2.28×10^{-3}	1.27×10^{-2}	4.59×10^{-2}	2.68×10^{-2}	2.38×10^{-3}
6	3.23×10^{-3}	1.11×10^{-2}	6.77×10^{-2}	2.67×10^{-2}	3.80×10^{-3}
7	2.80×10^{-3}	1.02×10^{-2}	1.01×10^{-1}	2.41×10^{-2}	2.25×10^{-3}
8	2.52×10^{-3}	1.07×10^{-2}	6×10^{-2}	1.83×10^{-2}	2.36×10^{-3}
9	2.07×10^{-3}	7.15×10^{-3}	5.56×10^{-2}	1.33×10^{-2}	1.82×10^{-3}
10	1.85×10^{-3}	7.22×10^{-3}	4.54×10^{-2}	6.29×10^{-3}	1.22×10^{-3}

barium carbonate. All determinations for radioactivity were made using barium carbonate plates and a "Nucleometer" windowless counter.

Phospholipid. The solvent from the initial extraction was distilled at reduced pressure in a current of nitrogen. The residue was taken up in dry petroleum ether and the phospholipid precipitated with acetone and magnesium chloride(7). The phospholipid was reprecipitated prior to assay for radioactivity. For radioactivity determinations, an aliquot of a petroleum ether solution of the phospholipid was transferred to a quartz combustion vessel, the solvent evaporated and the dried residue combusted in a copper oxide filled, quartz tube in a stream of air. The evolved carbon dioxide was collected and assayed as described above.

Cholesterol and fatty acids. The solvent was removed from the supernatant following precipitation of the phospholipid by distillation at low pressure in a stream of nitrogen. The residue was refluxed with a 15% solution of potassium hydroxide in alcohol for 48 hours. The cholesterol was then separated in several different ways. In one experiment, the cholesterol was precipitated as the digitonide directly from the basic saponification mixture. In another instance, the saponification mixture was acidified, extracted with ether and the ether solution was passed over an alumina column. The cholesterol and non-saponified material were eluted together with petroleum ether-benzene 9:1, the column was stripped with a solution of methanol-acetic acid 4:1. There was no detectable activity left on the alumina. The cholesterol was separated as the digitonide.

The most convenient method for separating the cholesterol and non-saponified material from the fatty acids was one involving the use of the ion exchange resin Amberlite IRA-400 (8). The saponification mixture was acidified and ether extracted. The ether extract was passed over a column of the resin; the non-acidic material passed directly through. The acids were eluted with a solution of ether-ethanol-conc. hydrochloric acid 70:30:8.

The cholesterol was precipitated from the neutral fraction as the digitonide. The cholesterol was recovered from the digitonides and recrystallized from acetone. Paper chromatography on "Quilon" impregnated paper(9) using methanol as the developing solvent gave R_f values between 0.53 and 0.58 for all 10 fractions. Radioautography was used(10) to determine R_f values and also to show that no radioactive contaminants were present. The cholesterol specific activities were determined by plating the cholesterol directly from an acetone solution and counting the plates in a "Nucleometer." The fatty acids obtained on elution were dried and converted to carbon dioxide by wet combustion.

The saponification procedure used did not give complete saponification in any case but one. In several instances the neutral residue remaining after precipitation of the choles-

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7. Sinclair, R. G., *ibid.*, 1930, v88, 575.

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9. Kritchevsky, D. and Calvin, M., *J. Am. Chem. Soc.*, 1950, v72, 4330.

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terol was subjected to a second saponification with 20% aqueous potassium hydroxide for 60 hours. Repetition of the ion exchange separation still gave some neutral material, which did not contain a digitonin precipitable component. The specific activities of the acids obtained upon elution corresponded closely with the activities of the acids obtained after the first saponification. In the cases of yolks 1, 2 and 7, where a second saponification was carried out, the specific activities were 7.37×10^{-4} μc/mg, 2.60×10^{-3} μc/mg, and 2.61×10^{-2} μc/mg, respectively.

Glycerol. The aqueous solution remaining after the saponification mixture had been extracted was distilled to dryness at reduced pressure. In several cases, the salt residue was extracted with hot pyridine and, after cooling, benzoyl chloride was added to this solution. Acidification and ether extraction yielded the glycerol tribenzoates of yolks 1, 2, 3, 4 and 6. These derivatives had melting points in the range 73-76° and none depressed the melting point of an authentic sample of this material.‡ For yolks 5, 7, 8, 9 and 10, the salt residue was extracted with hot acetone. Evaporation of the acetone in a current of nitrogen left the desired glycerol, which was also converted to the tribenzoate in a few cases. Wet combustion was used to assay radioactivity. In cases where a sample was assayed both as free glycerol and as tribenzoate the specific activities were in agreement.

Summary. (1) The distribution of radioactivity in the yolks of eggs laid by a hen fed sodium acetate-1-C^{14} has been determined. (2) The radioactivity appears in all fractions of all yolks in the following order of increasing specific activity: protein, glycerol, phospholipids, fatty acids, cholesterol.

The interest of Prof. Melvin Calvin during the course of this investigation is gratefully acknowledged.

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‡ We are indebted to Prof. James Cason for supplying a sample of this material.

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The Mechanisms of the Choline Oxidase and Transmethylase Enzyme Systems.* (19021)

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In an earlier investigation of the choline oxidase system(1) various requirements of the enzyme system for maximum activity in rat liver homogenates were studied. Since the work of Muntz(2) implied that in order for transmethylation from choline to homocysteine to take place choline must first be converted to betaine, an investigation of the role of all 3 possible donors, *i.e.*, choline, betaine aldehyde, and betaine, in forming methionine from homocysteine was undertaken.

Since previous work concerning transmethylation to homocysteine was carried out anaerobically(2,3), it appeared desirable to compare the roles of choline and its oxidation products in transmethylation both aerobically and anaerobically and to correlate oxidation data with transmethylation data. In the earlier study of the choline oxidase system(1), the results indicated that the oxidation of choline to betaine did not occur as smoothly as might be expected since after the first 10 minutes of choline oxidation betaine aldehyde oxidase became the rate-limiting enzyme in the system. Therefore, oxygen uptake data for oxidation of choline did not necessarily indicate that a mole of betaine was being formed for each mole of oxygen taken up. In order to study the actual mechanism of the reactions, the formation of betaine could be estimated indirectly by using a bicarbonate buffer and measuring acid production manometrically, provided suitable controls were employed. In this way the oxidation of choline and betaine aldehyde could be correlated with the formation of betaine and also with transmethylation if homocysteine was included in the system.

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

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Experimental. Adult male Holtzman rats weighing from 200 to 250 g were used as experimental animals. At the time of experiment the animals were sacrificed by decapitation, the livers removed *in toto*, and chilled in cracked ice. A portion of the liver was weighed out and homogenized in 5 volumes of ice-cold pH 7.8 bicarbonate buffer of the following composition: 0.039 M sodium bicarbonate and potassium chloride and 0.0033 M magnesium sulfate. One ml of homogenate was pipetted into previously prepared Warburg vessels. In the side arm of each of 16 Warburg vessels was added 0.5 ml 0.0234 M DL-homocysteine hydrochloride (General Biochemicals, Inc.) prepared in the bicarbonate buffer described above. 0.1 ml of water was added to the side arms of flasks 1, 2, 9, and 10. To the side arms of flasks 3, 4, 11, and 12 was added 0.1 ml 0.128 M choline chloride; of flasks 5, 6, 13, and 14, 0.1 ml of 0.128 M betaine aldehyde, prepared according to Voet (4); of flasks 7, 8, 15, and 16, 0.1 ml of 0.128 M neutralized betaine hydrochloride. The first 8 flasks were employed both for measuring oxidation of substrates and for determining carbon dioxide evolution due to betaine formation from choline and betaine aldehyde by the "direct method"(5). Therefore, 4 alternate flasks of the first 8 contained 0.2 ml 10% potassium hydroxide and the remaining 4 contained 0.2 ml water in the center wells. The last 8 flasks were employed for determining whether betaine could be formed anaerobically from either choline or betaine aldehyde. Since homocysteine can be desulfurated by rat liver homogenates with

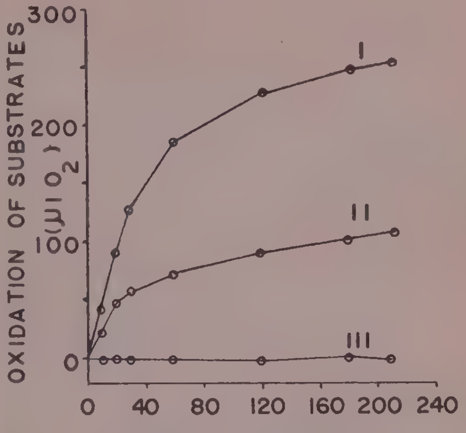

FIG. 1. The oxidation of equimolar amounts of choline (Curve I), betaine aldehyde (Curve II), and betaine (Curve III) by rat liver homogenate.

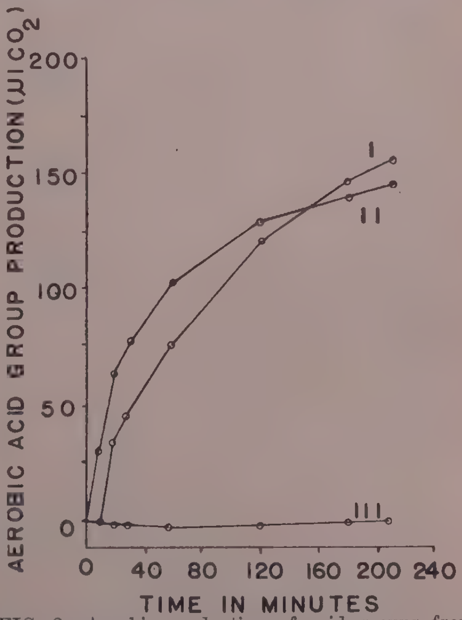

FIG. 2. Aerobic production of acid groups from choline (Curve I), betaine aldehyde (Curve II), and betaine (Curve III) by rat liver homogenate.

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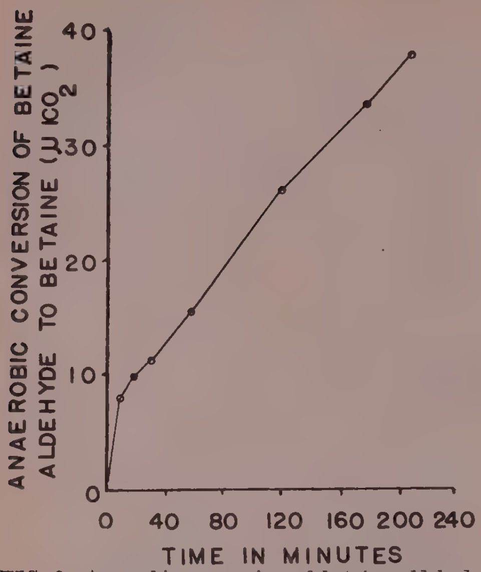

FIG. 3. Anaerobic conversion of betaine aldehyde to betaine by rat liver homogenate.

the formation of hydrogen sulfide(2), 0.2 ml of 10% cupric sulfate penta hydrate in 1 N sulfuric acid was added to the center wells of the last 8 flasks to absorb any hydrogen sulfide that might be liberated.

After the liver homogenate was added to the main compartment of the flasks, the last 8 flasks were gassed for 10 minutes with 95% nitrogen-5% carbon dioxide mixture. All flasks were then equilibrated on the Warburg bath at 37°C for 10 minutes after which the contents of the side arms were added to the homogenate and manometer readings taken at intervals for 210 minutes. The methionine present in each flask after the 210-minute period was estimated according to the method outlined by Dubnoff(3).

Results. Manometric studies. The data obtained manometrically have been analyzed as shown in Fig. 1 to 3 and Table I. The data presented are an average of the results from 10 animals although each animal conformed to the same pattern. In Fig. 1 the oxygen uptake data reveals that at the end of 210 minutes the oxidation of choline and betaine aldehyde approached the theoretical values of 286 and 143 μl O_2, respectively, assuming that the oxidation of choline to betaine requires 1 mole of oxygen and that of betaine aldehyde, 0.5 mole of oxygen. The oxidation of betaine was zero, which also implies that the methionine formed from homocysteine was not oxidized in the system. In Fig. 2 the calculated carbon dioxide evolution due to formation of acid (assumed to be betaine) from the substrates added indicates that betaine aldehyde was oxidized directly to betaine as shown by the absence of a lag in the curve. However, for the first 10 minutes choline did not give rise to betaine as shown by the lack of acid production for the first 10-minute period. After 210 minutes the total acid production both from choline and betaine aldehyde approached approximately the same value. However, the actual amount of betaine formed from both substrates as measured by carbon dioxide production was only one-half the theoretical value (286 μl CO_2). Therefore, comparing Fig. 1 and 2, the carbon dioxide evolution from choline should equal the oxygen uptake and that from betaine aldehyde should be twice the oxygen uptake, assuming the following reactions are the only ones involved:

Choline $\xrightarrow{+\frac{1}{2}O_2}$ betaine aldehyde $\xrightarrow{+\frac{1}{2}O_2}$ betaine (1 mole choline + 1 mole O_2 $\longrightarrow$ 1 mole betaine, or 1 CO_2; 1 mole betaine aldehyde + ½ mole O_2 $\longrightarrow$ 1 mole betaine, or 1 CO_2).

In the anaerobic experiments no measurable production of betaine from choline was

TABLE I. Ratio of Carbon Dioxide Evolution to Oxygen Consumption (Betaine Formation to Substrate Oxidized).

	$CO_2 : O_2$			
	Choline		Betaine aldehyde	
Time (min)	Uncorrected*	Corrected*	Uncorrected*	Corrected*
10	0	0	1.50	1.10
20	.41	.29	1.35	1.13
30	.36	.27	1.37	1.18
60	.41	.33	1.44	1.20
120	.53	.41	1.37	1.10
180	.59	.46	1.38	1.05
210	.60	.47	1.34	1.03
Theoretical ratio	1		2	

* See text for explanation of terms.

TABLE II. Methionine Formation and Gas Exchanges Under Aerobic and Anaerobic Conditions from Homocysteine and the Substrates Listed After 210 Minutes.

Gas phase	Substrate	Methionine formed (μ moles)	Oxygen consumed (μ moles)	CO_2 evolved (betaine formed) (μ moles)
Air	Choline	.40	11.4	7 (5.3 corrected)*
	Betaine aldehyde	.40	4.8	6.4 (4.8 '')*
	Betaine	.40	0	0
95% N_2–5% CO_2	Choline	.10	0	0
	Betaine aldehyde	.36	0	1.7
	Betaine	1.32	0	0

* See text for explanation of term.

observed as shown in Fig. 3. However, with betaine aldehyde as substrate, a marked production of betaine was observed anaerobically as demonstrated by the curve for betaine aldehyde in Fig. 3. Therefore, it appears that 2 pathways exist for conversion of betaine aldehyde to betaine, one requiring oxygen and the other proceeding anaerobically. The anaerobic pathway which may be a dismutation reaction, appears to be approximately one-third as active in rat liver homogenates as the aerobic pathway as shown by the relative rates of conversion of betaine aldehyde to betaine.

In Table I are presented the ratios of carbon dioxide evolution (or betaine formation) to oxygen uptake for choline and betaine aldehyde at the various time intervals. The ratios have been calculated in 2 ways: the ratios actually obtained and the ratios corrected for anaerobic conversion of betaine aldehyde to betaine assuming that the anaerobic pathway is still active under aerobic conditions. The latter method is probably more meaningful since the carbon dioxide generated by the aerobic conversion of the substrate to betaine should be compared to the oxygen uptake involved in conversion. The calculations indicate, therefore, that the ratios for choline approach 0.50 and for betaine aldehyde 1.00, both of which are one-half the theoretical ratios. Therefore, only one-half as much betaine is formed from either substrate as expected. The reason for the low yield of betaine from both choline and betaine aldehyde is not clear although one might surmise several possible explanations. One of them which would fit the data obtained assumes an instantaneous dimerization of betaine aldehyde at the pH employed, 7.8.

Transmethylation studies. The results of the methionine analyses after the 210-minute incubation period are presented in Table II. In order to compare the manometric results with formation of methionine, the final oxygen uptake and carbon dioxide evolution data are reported in the table as micromoles of oxygen consumed or carbon dioxide evolved and the methionine production as micromoles of methionine formed. It can be observed from the table that aerobically choline, betaine aldehyde, and betaine are equivalent in transfer of a methyl group to homocysteine. Also there appears to be no correlation either with the oxidation of substrates or with the formation of betaine since aerobically a large excess of both choline and betaine aldehyde are oxidized to betaine over the amount actually involved in transmethylation. That the methionine formed is not oxidatively removed is borne out by the fact that no oxygen uptake was observed in the flask containing betaine as substrate (Fig. 1) even though methionine was formed.

The anaerobic results indicate that methyl donation by choline occurs only to a negligible extent, which is probably due to lack of conversion of choline to betaine. However, betaine aldehyde, since it is anaerobically converted to betaine, does contribute methyl groups to homocysteine though not as greatly as betaine itself. Moreover, the anaerobic transfer of methyl groups from betaine is significantly greater than the aerobic transfer.

Summary. (1) The mechanism of choline and betaine aldehyde conversion to betaine and methyl group transfer to homocysteine have been investigated both in aerobic and anaerobic systems. (2) The expected yield of acid groups, assumed to be betaine, from

oxidation of choline and betaine aldehyde is only one-half the theoretical. (3) Betaine aldehyde appears to be metabolized by 2 pathways, one an aerobic oxidation and the other an anaerobic conversion to betaine. (4) Choline, betaine aldehyde, and betaine are equivalent methyl group donors to homocysteine under aerobic conditions. Anaerobically choline is almost inactive in furnishing methyl groups to homocysteine. Betaine, however, is a more active methyl donor anaerobically than under aerobic conditions.

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Effect of Aminopterin on Choline, Betaine Aldehyde, and Betaine Metabolism.* (19022)

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In several recent publications(1-3) it has been reported that dietary aminopterin causes a marked decrease in tissue choline oxidase activity. This decrease in activity has been reversed by incubating the liver homogenates with combinations of several of the presumed precursors of the *Leuconostoc citrovorum* (LCF) factor, namely, ascorbic acid, folic acid, vit. B_{12}, and LCF itself(1,2). Moreover, it has been shown from this laboratory that dietary aminopterin induces a fault in the ability of rats to synthesize ascorbic acid(4). The sum of the preceding work, therefore, indicates that folic acid and ascorbic acid metabolism are disturbed by dietary aminopterin, and that this may be the basic cause of the decrease in choline oxidase activity. Since the choline oxidase(5) and betaine aldehyde oxidase(6) systems are believed to be intimately connected with the transmethylation reactions of homocysteine, and since a deficiency of dietary folic acid has been shown to disturb this transmethylation reaction in chickens(7), the present investigation was undertaken to determine the extent of the disturbance of the transmethylation reactions involving choline, betaine aldehyde and betaine by dietary aminopterin in rats.

In a preceding publication(6), a study of the mechanism of the metabolism of choline and betaine aldehyde has shown that the latter metabolite can be converted to betaine either aerobically or anaerobically, while choline oxidase is entirely an oxygen-requiring system. Because of the close relationships of these metabolites with transmethylation, it appeared desirable to study the effects of dietary aminopterin on the different aspects of choline, betaine aldehyde, betaine, and methionine metabolism uncovered in the preceding investigation.

Experimental and results. Adult male rats of the Holtzman strain were employed as experimental animals. One group of rats was fed a normal synthetic ration containing 18% casein as previously described(1). Other animals were fed the same ration except that 4 mg of aminopterin† were included per kilo

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† The author wishes to thank Dr. J. J. Oleson of Lederle Laboratories, Pearl River, N. Y., for the generous supply of aminopterin used in these studies.

TABLE I. Effect of Dietary Aminopterin on Aerobic Oxidation of Choline and Betaine Aldehyde, Anaerobic Dehydrogenation of Betaine Aldehyde, and Formation of Methionine in Rat Liver Homogenates.

	Substrate	Microliters of gas exchanged (μl O_2)		Methionine formed per 210 min per g liver (μg)	
		I*	II*	I*	II*
Aerobic results	Choline	255	172	354	40†
	Betaine aldehyde	107	72	354	80†
	Betaine	0	0	354	112
		(μl CO_2)		(μg)	
Anaerobic results	Choline	0	0	90†	9†
	Betaine aldehyde	39	27	320	167
	Betaine	0	0	770	530

* I = Avg of 10 rats fed normal ration; II = avg of 8 rats fed normal ration + aminopterin.

† These figures must be considered negligible amounts since they are below the range of accuracy of the method.

of ration. After about 10 days deficiency symptoms suddenly became marked in the latter animals and death resulted within a few hours after the onset of these symptoms. For this reason several groups of aminopterin-fed rats had to be set up in order to obtain enough animals before death for the enzyme studies. When the effects of the aminopterin became strongly evident, the animals were sacrificed for determination of the effect of the anti-vitamin upon the oxidation of choline and betaine aldehyde, the anaerobic conversion of betaine aldehyde to betaine, and the aerobic and anaerobic formation of methionine from choline, betaine aldehyde, and betaine. The experimental methods are the same as those indicated in the preceding paper(6).

The average results of the aerobic oxidation of choline and betaine aldehyde, the anaerobic dehydrogenation of betaine aldehyde, and the aerobic and anaerobic formation of methionine in homogenates of the 2 groups of rats are presented in Table I. The results have been included in a single table for easier study of the data as a whole. There was no overlapping of the corresponding results for the 2 groups of animals.

From the results it can be seen that the oxidation of choline is depressed by dietary aminopterin as has been previously reported (1-3). Both the aerobic oxidation and anaerobic dehydrogenation of betaine aldehyde are also depressed by the anti-vitamin. This raises the question of whether the first enzyme, *i.e.*, choline oxidase, or the second, betaine aldehyde oxidase, is responsible for the decrease in oxygen uptake by aminopterin when choline is the substrate. The results reported in Table I for choline oxidase activity probably partially reflect the lowered betaine aldehyde oxidase activity since the figures reported are the total oxygen uptakes for the 210-minute period. However, choline oxidase activity, when measured at the end of the first 10 minutes after addition of choline, is not influenced to an appreciable extent by the slower second reaction (betaine aldehyde oxidase) as has been pointed out in earlier work(6). Also the depression of choline oxidase activity by dietary aminopterin can be demonstrated by data taken from the first 10 minutes of the choline oxidase assay. Therefore, even though betaine aldehyde oxidase is decreased by aminopterin, the similar effect on choline oxidase does not appear to be an artifact.

It is also interesting to observe that the anaerobic conversion of betaine aldehyde to betaine is decreased by dietary aminopterin. Therefore, it appears that the entire mechanism for converting choline or betaine aldehyde to the actual methyl donor, betaine, is disturbed by interfering with folic acid metabolism.

The data representing the formation of methionine under the various conditions presented in the table indicate that the transmethylating mechanism is also depressed by dietary aminopterin. In some cases, *i.e.*, in the aerobic transmethylation from choline and betaine aldehyde and anaerobic transmethylation from choline, the formation of methionine in the aminopterin-fed rats was decreased so low it could not be measured.

It is interesting to note that in most cases the gas exchanges and formation of methionine for the rats fed aminopterin appear to be decreased in a constant ratio with respect to the normal controls. The ratios of the results for the aminopterin-fed rats to those for the normal rats in the oxidation of choline and betaine aldehyde, the anaerobic conversion of the aldehyde to betaine, and the anaerobic formation of methionine tend to approach a common value of about 0.6. Therefore, it appears that a factor common to each of these enzyme systems is disturbed by dietary aminopterin. It remains to be seen what the common factor is, although it is undoubtedly concerned with the metabolism of folic acid.

Summary. 1. The effects of dietary aminopterin upon the aerobic and anaerobic metabolism of choline, betaine aldehyde, and betaine have been investigated. 2. Oxidation of choline and betaine aldehyde, anaerobic conversion of betaine aldehyde to betaine, and both aerobic and anaerobic transmethylation to homocysteine are decreased significantly by dietary aminopterin.

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System for Measuring and Designating Antigenic Components of Influenza Viruses with Analyses of Recently Isolated Strains. (19023)

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Previous investigations(1,2) in this laboratory to examine the antigenic composition of influenza viruses have been extended, in the current study, to include the A prime agents which were present during 1950-51, a number of WS- and PR8-like (type A) strains isolated in recent years, and the influenza C viruses. A system for measuring and designating certain antigenic components of influenza viruses has been developed on the basis of these findings and is presented here. The new system promises to be especially useful for those investigations in the fields of epidemiology and preventive medicine in which definitive information on the antigenic relationships of newly isolated strains of influenza is required quickly.

Materials and methods. Virus strains. Pertinent information concerning the history of certain of the viruses used in the present study is given in Table I. The Syr II(3), Robert and Saunders strains were obtained from Dr. Bernice Eddy. Bur 99 and the Ba, Nou, Chantal and Fa viruses(4) were furnished by Dr. P. Lepine. The HSC-18, HSC-19 and HSC-20 agents, isolated from infants at Toronto, Canada, were supplied by Dr. C. E. van Rooyen. Dr. F. P. Nagler sent the Bent I and Bent 2 strains and Dr. C. H. Andrewes supplied the London-1-51, England-1-51 and Eire-1-51 viruses. Strains Ann Arbor-1-51 and Ann Arbor-2-51 were obtained from Dr. T. Francis, Jr., and Dr. G. Meikeljohn furnished the Be-1-51 virus. Strain Bonoan, isolated at the Fourth Army Area Medical Laboratory, Fort Sam Houston, Texas, was obtained from Major L. V. Murphy, V.C., U.S.A., and the SWE-3-50 virus was sent by Dr. A. Isaacs. The Greenland-3-51, FM-10-51, FM-111-51 and Dion agents were isolated in this laboratory. The Benson strain was obtained from Dr. J. Salk. The remaining A-type viruses as well as the B strains em-

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TABLE I. Data on Strains of Influenza A and C Included in Studies.

Type	Strain	Year isolated	Passage history	Origin
A	WS	1933	F?/M?/E5	England
	PR8 (ISSC)*	34	F1/M 691/E7-8	Puerto Rico
	FM1 (ISSC)*	47	M7/E15-17	New Jersey
	Syr II	47	>E1	New York
	Ba	49	>E1	France
	Nou	49	>E1	"
	Chantal	49	>E1	"
	Fa	49	>E1	"
	CAW-1	49	E3	Victoria Island, Canada
	-2	49	E3	" " "
	-3	49	E3	" " "
	Canadian Arctic 1A	49	E3	" " "
	HSC-18	50	E5	Ontario, Canada
	-19	50	E4	" "
	-20	50	E5	" "
	Benson	50	E4	Pennsylvania
	FW-1-50	50	E3-4	Wyoming
	Bent 1	50	E4	Alberta, Canada
	2	50	E4	" "
	SWE-3-50	50	>E1	Sweden
	London-1-51	51	E4-6	England
	Saunders	51	E3	N. Y. (English ship)
	Robert	51	E4	N. Y. (French ship)
	Eire-1-51	51	>E1	Ireland
	Bur 99	51	>E1	France
	Greenland-3-51	51	E2-4	Greenland
	FM-10-51	51	E3-5	New Jersey
	FM-111-51	51	E3-4	" "
	Dion	51	E2	Washington, D. C.
	Ann Arbor-1-51	51	E4	Michigan
	-2-51	51	E3	"
	Bonoan	51	E3	Texas
	Be-1-51	51	F2/E4	California
	England-1-51	51	E3	England (Liverpool)
C	1233	47	E50-51	New York
	JJ	50	E22-23	Michigan

F = ferret, M = mouse, E = egg.

* Standard diagnostic strains of the Influenza Strain Study Center, Commission on Influenza, Armed Forces Epidemiological Board.

ployed in the current work were described in a previous report(2). Influenza C strains 1233(5) and JJ(6) were received from Drs. R. M. Taylor and T. Francis, Jr., respectively.

Sera. Roosters weighing 5 to 8 lb were usually injected on one occasion with 5.0 ml of antigen intravenously and with 5.0 or 10.0 ml intraperitoneally. Crude infected allantoic fluid, freshly harvested, served as antigen. Certain of the recently isolated A prime strains did not yield chorioallantoic fluids of sufficient antigenic potency to evoke a satisfactory antibody response when the above procedure was employed. Hence, when the hemagglutinin titer of the fluid was less than 1:640 (fluids with titers less than 1:160 were not used), similar amounts of antigen were administered on 2 successive days. The strain specificity of the sera was not detectably influenced by either the volume of antigen used or by the number of injections within the limits defined above. The roosters were bled 10 days following the initial injection and their sera inactivated and stored in dried form until used in the tests. Sera against strains SWE-3-50, FM-111-51, Eire-1-51 and Bur 99 titered 1:400 with homologous antigen; all other sera titered 1:800 or greater. None of the sera obtained from the roosters prior to immunization contained demonstrable antibody for influenza virus.

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5. Taylor, R. M., *Am. J. Pub. Health*, 1949, v39, 171.

6. Francis, T., Jr., Quilligan, J. J. Jr., and Minuse, E., *Science*, 1950, v112, 495.

Hemagglutination technic. The hemagglutination and hemagglutination-inhibition titrations were performed in the manner described earlier(2). The antigens were preserved by drying from the frozen state(7) and were rehydrated within 24 hours of their use in the tests. The antigens for the day's work were diluted 1:10 in physiological saline solution and allowed to stand for 1 hour at room temperature before titrating to permit elution of virus from particulate matter in the suspension. These procedures for handling antigen were important. Thus, storage of antigen in the desiccated state prevented the formation of precipitate, with resultant viral aggregation which occurs when allantoic fluids are refrigerated in the fluid state; furthermore, the period of storage at room temperature assisted elution and provided maximal viral dispersion. Under such carefully controlled conditions, the variations in serum titer resulting from viral aggregation (avidity factor of Hirst)(8) were minimized and the inhibitory titers were generally reproducible with regularity; hence, in these studies, we regarded differences in endpoints of a single 2-fold dilution of serum to be the usual range of experimental error when the serum was tested with the same or different antigens prepared from the same strain of virus. In the present study, as in the earlier ones(1,2), the serum titers were adjusted to give a 1:800 titer with homologous virus so that the results with the individual sera could be compared more easily.

Results. Analysis of some 1950-51 A prime viruses. Table II gives an interpretive summary of the results of tests to determine the antigenic patterns of a group of A prime viruses isolated during 1950 and 1951 and to compare these strains with 4 prototype viruses: WS, PR8, FM1 and FW-1-50 (see below for discussion on prototype strains). In the table, serum inhibitory titers of 1:800 or 1:400 are represented by 3, 1:200 by 2, 1:100 or 1:50 by 1 and less than 1:50 by 0. In several instances, the adjusted serum titer obtained in tests with heterologous antigen was 1:1600,

TABLE II. Interpretive Summary of Agglutination-Inhibition Tests Showing Relationships of 1950-51 Influenza A Strains to Prototype Strains.

	Antigens																			
Rooster antisera	WS England 1933	PR8 Puerto Rico 1934	FM1 N. J. 1947	Benson Penn. 1950	FW-1-50 Wyoming 1950	London-1-51 1951	Saunders, English ship at N.Y. 1951	Robert, Fr. ship from England-51	Eire-1-51 1951	Burr 99 France 1951	Greenland-3-51, 1951	FM-10-51 N. J. 1951	FM-111-51 N. J. 1951	Dion Wash., D.C. 1951	Ann Arbor-1-51 Mich. 1951	Ann Arbor-2-51 Mich. 1951	Bonoan Texas 1951	BE-1-51 California 1951	SWE-3-50 Sweden 1950	England-1-51 Liverpool 1951
WS 1933	*3**	2	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
PR8 1934	1	*3*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FM1 1947	0	0	*3*	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Benson 1950	0	1	3	*3*	1	1	ND†	ND	ND	ND	ND	1	ND	ND	ND	ND	ND	ND	1	ND
FW-1-50 1950	0	1	3	3	*3*	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
London-1-51 1951	0	1	3	3	3	*3*	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Eire-1-51 1951	0	0	3	3	3	3	3	3	*3*	3	3	3	3	3	3	3	3	3	3	3
Bur 99 1951	0	0	3	3	2	3	3	3	3	*3*	3	3	3	3	3	3	3	3	3	3
Greenland-3-51 1951	0	0	3	3	3	3	3	3	2	3	*3*	3	3	3	3	3	3	3	3	3
FM-10-51 1951	0	1	3	3	3	3	3	3	2	3	3	*3*	3	3	3	3	3	3	3	3
FM-111-51 1951	0	0	3	3	3	3	3	3	3	3	3	3	*3*	3	3	3	3	3	3	3
SWE-3-50 1950	0	0	1	2	2	3	3	3	3	3	3	3	3	3	2	3	3	3	*3*	3
England-1-51 1951	0	0	2	3	3	3	3	3	1	3	3	3	3	3	3	3	3	3	3	*3*

* 3 = titer 1:800-1:400; 2 = 1:200; 1 = 1:100-1:50; 0 = <1:50. † ND = not done.

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7. Hilleman, M. R., Buescher, E. L., and Smadel, J. E., 1951, *Public Health Rep.*, in press.

8. Hirst, G. K., *J. Exp. Med.*, 1943, v78, 407.

TABLE III. Interpretive Summary of Agglutination-Inhibition Tests Showing Relationships of WS- and PR8-Like Viruses from Recent Outbreaks to Prototype and 1951 Strains.

	Antigens																
Rooster antisera	WS England 1933	Syr II N. Y. 1947	PR8 Puerto Rico 1934	Ba France 1949	Nou France 1949	Chantal France 1949	Fa France 1949	HSC-18 Canada 1950	HSC-19 Canada 1950	HSC-20 Canada 1950	CAW-1 Canada Arctic 1949	CAW-2 Canada Arctic 1949	CAW-3 Canada Arctic 1949	CA-1A Canada Arctic 1949	FM1 N. J. 1947	FW-1-50 Wyoming 1950	FM-10-51 N. J. 1951
WS, 1933	*3*	3	2	2	2	2	2	1	1	1	1	1	1	0	1	0	0
Syr II, 1947	1	*3*	1	ND*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0	0	0
PR8, 1934	1	0	*3*	3	3	3	3	3	3	2	2	2	2	2	0	0	0
Ba, 1949	0	ND	3	*3*	3	3	3	3	3	3	2	2	2	2	0	0	0
Chantal, 1949	0	ND	3	3	3	*3*	3	2	2	2	2	2	2	1	0	0	0
HSC-18, 1950	1	ND	3	3	3	3	3	*3*	3	3	3	3	3	3	0	0	0
HSC-19, 1950	0	ND	3	3	3	3	3	3	*3*	3	3	3	3	3	0	0	0
CAW-1, 1949	1	ND	3	3	3	3	3	3	3	3	*3*	3	3	3	1	0	0
CAW-3, 1949	0	0	3	3	3	3	3	3	3	3	3	3	*3*	3	0	0	0
CA-1A, 1949	2	ND	3	3	3	3	3	3	3	3	3	3	3	*3*	1	0	0
FM1, 1947	0	0	0	0	0	0	0	0	0	0	0	0	0	0	*3*	1	1
FW-1-50, 1950	0	0	1	1	1	1	0	2	2	1	1	0	0	1	3	*3*	3

* ND = not done.

i.e., 2 times as high as with homologous antigen (1:800). This variation was considered to be within the range of experimental error of the test and the 1:1600 titer was counted as 3 in the table.

It is readily apparent in the table that except for the Benson agent, the 1950-51 viruses comprised a remarkably homogenous antigenic group, as determined by this method, and that they closely resembled the FW-1-50 strain (sometimes designated as the Cuppett strain). It is also apparent that these viruses reacted poorly with FM1 antiserum and gave no significant reaction with the WS (Ann Arbor-1-51 is an exception) or PR8 antiserum. Slight deviations from the general pattern such as those displayed by the SWE-3-50 serum when tested with FM1 antigen and by the England-1-51 serum when crossed with Eire-1-51 antigen were confirmed in repeated tests. The Benson agent isolated at Pittsburgh in 1951 stood out in striking contrast to the other 1950-51 agents; the former virus was essentially indistinguishable from the FM1 agent isolated in 1947. The antigenic relationships of the prototype viruses, *i.e.*, WS, PR8, FM1 and FW-1-50, to each other were essentially the same as previously reported(2).

Analysis of some recently isolated WS- and PR8-like viruses. During the 1947-1950 period, a small number of viruses were isolated which closely resembled the WS(9) and PR8(10) agents and showed little or no relationship to the A prime strains which were predominant during this period. The results of tests to determine the antigenic patterns of a number of these viruses in comparison with prototype strains are summarized in interpretive form in Table III. It is apparent that the 4 French viruses Ba, Nou, Chantal and Fa, the HSC-18, 19 and 20 viruses (isolated from infants in Toronto) and the CAW-1, 2, 3 and CA-1A strains were quite similar to each other and that these agents closely resembled the PR8 strain. Syr II virus was related to the WS agent as revealed by its reaction with antiserum to the latter although

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9. Smith, W., Andrewes, C. H., and Laidlaw, P. P., *Lancet*, 1933, v2, 66.

10. Francis, T., Jr., *Science*, 1934, v80, 457.

the WS antigen reacted poorly with the Syr II serum.

Analysis of influenza C strains. Influenza C strains 1233 and JJ examined in the usual manner were indistinguishable.

System for measuring and designating antigenic components. The early demonstration (11) of antigenic heterogeneity among strains of influenza A was followed by the observation (12-14) that such polyvalency resulted from both qualitative and quantitative variations in a number of ill-defined and irregularly distributed antigenic components. These antigenic qualities appeared as shared constituents and it became apparent that the separation of these agents into precise subgroups was impossible. In attempting any systematic arrangement of this group of agents, it would appear desirable, *a priori*, to define the antigenic constitution of each strain based on the number and kind of available antigenic components as suggested by Friedewald(15) but practical considerations apparently preclude the attainment of this ideal at the present time. The need for a more simple approach is clearly evident and a method for the measurement and symbolic expression of antigenic (hemagglutinating) constitution of influenza viruses is presented here.

It was shown previously(2) that the A-type viruses comprised a graded series of antigenic forms in which precise subgrouping was impossible. It was significant, however, that all the viruses examined reacted to marked degree with 1 or more of the antisera to the WS, PR8, FM1 and FW-1-50 strains; thus antibodies to at least some of the antigens of each virus examined were represented in the 4 sera. These 4 strains have been designated prototype viruses. Within the B group, all the strains reacted with antisera to the Lee or

TABLE IV. Antigenic Formulae of Some Influenza A, B and C Viruses.

Strain	Year isolated	Formula
		(WS) (PR8) (FM1) (FW150)
WS	1933	$A\ a_3\ b_1\ c_0\ d_0$
Syr II	47	$A\ a_3\ b_0\ c_0\ d_0$
PR8 (ISSC)	34	$A\ a_2\ b_3\ c_0\ d_1$
Ba	49	$A\ a_2\ b_3\ c_0\ d_1$
Nou	49	$A\ a_2\ b_3\ c_0\ d_1$
Chantal	49	$A\ a_2\ b_3\ c_0\ d_1$
Fa	49	$A\ a_2\ b_3\ c_0\ d_0$
CAW-1	49	$A\ a_1\ b_2\ c_0\ d_1$
2	49	$A\ a_1\ b_2\ c_0\ d_0$
3	49	$A\ a_1\ b_2\ c_0\ d_0$
CA-1A	49	$A\ a_0\ b_2\ c_0\ d_1$
HSC-18	50	$A\ a_1\ b_3\ c_0\ d_2$
19	50	$A\ a_1\ b_3\ c_0\ d_2$
20	50	$A\ a_1\ b_2\ c_0\ d_1$
Weiss	43	$A\ a_2\ b_2\ c_2\ d_0$
Henry	36–7	$A\ a_1\ b_3\ c_3\ d_2$
Hyde	35	$A\ a_0\ b_3\ c_3\ d_2$
Hume	47	$A\ a_0\ b_2\ c_3\ d_0$
Hickcox	40–1	$A\ a_1\ b_1\ c_3\ d_2$
CAW-5	49	$A\ a_2\ b_0\ c_3\ d_3$
FM1	47	$A\ a_1\ b_0\ c_3\ d_3$
Benson	50	$A\ a_1\ b_0\ c_3\ d_3$
Wilfong	48	$A\ a_0\ b_0\ c_3\ d_3$
4ML-48/49	48–9	$A\ a_0\ b_0\ c_1\ d_3$
SF-1-49	49	$A\ a_0\ b_0\ c_1\ d_3$
FW-1-50	50	$A\ a_0\ b_0\ c_1\ d_3$
Bent 1	50	$A\ a_0\ b_0\ c_1\ d_3$
2	50	$A\ a_0\ b_0\ c_1\ d_3$
Berkeley-1-50	50	$A\ a_0\ b_0\ c_1\ d_3$
AH-1-50	50	$A\ a_0\ b_0\ c_1\ d_3$
SWE-3-50	50	$A\ a_0\ b_0\ c_1\ d_3$
London-1-51	51	$A\ a_0\ b_0\ c_1\ d_3$
Saunders	51	$A\ a_0\ b_0\ c_1\ d_3$
Robert	51	$A\ a_0\ b_0\ c_1\ d_3$
Eire-1-51	51	$A\ a_0\ b_0\ c_1\ d_3$
Bur-99	51	$A\ a_0\ b_0\ c_1\ d_3$
Greenland-3-51	51	$A\ a_0\ b_0\ c_1\ d_3$
FM-10-51	51	$A\ a_0\ b_0\ c_1\ d_3$
FM-111-51	51	$A\ a_0\ b_0\ c_1\ d_3$
Dion	51	$A\ a_0\ b_0\ c_1\ d_3$
Ann Arbor-1-51	51	$A\ a_1\ b_0\ c_1\ d_3$
Ann Arbor-2-51	51	$A\ a_0\ b_0\ c_1\ d_3$
Bonoan	51	$A\ a_0\ b_0\ c_1\ d_3$
Be-1-51	51	$A\ a_0\ b_0\ c_1\ d_3$
England-1-51	51	$A\ a_0\ b_0\ c_1\ d_3$
		(Lee) (Warner)
Lee	40	$B\ a_3\ b_0$
Gt. Lakes A	50	$B\ a_3\ b_0$
Gt. Lakes B	50	$B\ a_3\ b_0$
Gt. Lakes C	50	$B\ a_3\ b_0$
Hawaii	44–5	$B\ a_1\ b_3$
Meguro	50	$B\ a_1\ b_3$
Pooh	50	$B\ a_1\ b_3$
Seattle-2-49	49	$B\ a_1\ b_3$
Czech	45	$B\ a_1\ b_3$
Warner	48	$B\ a_1\ b_3$
L1-B-50	50	$B\ a_1\ b_3$
MB	50	$B\ a_1\ b_3$
IB1	50	$B\ a_0\ b_3$
		(1233)
1233	47	$C\ a_3$
JJ	50	$C\ a_3$

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11. Magill, T. P., and Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1936, v35, 463.

12. Burnet, F. M., *Australian J. Exp. Biol. and Med.*, 1937, v15, 369.

13. Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, v19, 273.

14. Smith, W., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1938, v19, 293.

15. Friedewald, W. F., *J. Exp. Med.*, 1944, v79, 633.

Warner strains(2) which have been designated the prototype viruses for this group. The results of tests with influenza C viruses are mentioned in this report and strain 1233 is considered the prototype.

Based on the degree of reaction with the prototype antisera, the antigenic formulae of a number of influenza A, B and C viruses have been constructed and are presented in Table IV. In the table, the strain type A, B or C is designated by the appropriate capital letter. Within the A group, WS serum is designated by small letter a, PR8 by b, FM1 by c and FW-1-50 by d; in the B group Lee is represented by a and Warner by b. Strain 1233 is designated a in the C group. The extent of reaction of a given virus in terms of its inhibition by the various antisera is designated by numbers 0, 1, 2 or 3 which represent the same titers as do the numerical values used in Tables II and III. To illustrate, Table II shows that type A WS virus gave a 3 reaction with prototype serum a (WS), 1 reaction with serum b (PR8), 0 reaction with c (FM1) and 0 with d (FW-1-50), giving the assembled formula A $a_3b_1c_0d_0$ for the WS strain. Similarly, type A London-1-51 virus titered 0 with serum a, 0 with b, 1 with c and 3 with d giving the formula A $a_0b_0c_1d_3$. Certain of the strains for which formulae are given in Table IV were analyzed and their relationships presented in a previous report(2) in which a different system for designating serum titers was used. A 4+ or 3+ value by the earlier method is equivalent to 3 by the present system, 2+ to 2, 1+ or ± to 1 and 0 to 0. It is to be noted that the antigenic formulae presented in Table IV are in minor disagreement in certain instances, with the formulae calculated from the preliminary data given earlier(2). Thus, the WS formula would be A $a_3b_0c_0d_0$ on the basis of the previous analyses instead of A $a_3b_1c_0d_0$ as given in Table IV. These differences were due to the 2-fold variation in serum titers which may be obtained in repeated tests with a given serum and virus. In effect, then, the formula gives the antigenic type of virus and designates whether there was strong, moderate, weak, or no reaction with each of the prototype antisera. It is apparent that the same general relationships which were shown in the interpretive summaries are revealed in the antigenic formulae.

Discussion. The infrequent appearance of WS- and PR8-like viruses during the period since 1947 stands in marked contrast to the overwhelming occurrence of agents of the A prime form. The reappearance of earlier antigenic forms was observed to occur among the A prime group as well as the A type WS- and PR8-like agents; thus the 1951 Benson virus was essentially identical to the 1947 FM1 agent in spite of the apparently continuous and progressive antigenic change which the A prime viruses have shown during this period(1,2).

The question has been raised as to whether the older forms really reappear as disease-producing agents in patients or whether they are found as the result of laboratory contamination(16). The question is important since the answer will materially influence our thinking with regard to the need for including the older antigenic forms in the current standard influenza vaccine. It may be of some significance that most of the recently isolated PR8-like viruses were recovered from persons who might be expected to have little or no immunity for influenza. Thus, the CAW and Canadian Arctic strains(17,18) originated from Eskimos in a remote and isolated region in which the general antibody level for influenza virus was low. The HSC strains were from children less than 2 years of age who may constitute a virgin population insofar as influenza is concerned. The French(19) strain Chantal came from an infant 1 year of age and Ba from a child somewhere between the ages of 3 and 10 (no record of exact age). Nou virus was isolated from a

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16. Andrewes, C. H., and Isaacs, A., United Nations, World Health Organization, *Preliminary Report on Influenza Strains Isolated in* 1950-51, 1951, June 20.

17. van Rooyen, C. E., McClelland, L., and Campbell, E. K., *Canadian J. Pub. Health,* 1949, v40, 447.

18. Nagler, F. P., van Rooyen, C. E., and Sturdy, J. H., *Canadian J. Pub. Health,* 1949, v40, 457.

19. Lepine, P., 1951, Personal communication.

pool of throat washings from a child 8 years of age and a nurse 40 years old; the nurse cared for the Chantal infant in a ward which was located near that of the 8-year-old child. Strain Fa was from an adult 50 years of age. It is our opinion that the weight of evidence strongly suggests that PR8-like strains have produced occasional human disease in the last few years. The infrequency of such apparent infections may be dependent more on the continued presence of relatively high levels of antibody against PR8 viruses in most populations than on the virtual disappearance of the virus from the face of the earth. It is likely that a strain of influenza virus for which the majority of the population possess antibodies in appreciable amounts has a low epidemic potentiality and that such a strain contributes relatively little to the prophylactic value of a polyvalent influenza vaccine.

It seems desirable that any system for the serological typing and systematic arrangement of influenza strains should be simple and rapid of performance and should give reasonably reproducible results. In addition, the method employed should provide definitive information concerning not only major differences but minor dissimilarities as well. These requirements seem to be met by the agglutination-inhibition test using chicken antiserum and the system of antigenic formulation described.

The results of the inhibitory tests were highly reproducible not only in repeated assay of a given virus antigen and antiserum but also in tests with the sera from 2 birds immunized with the same virus(2); thus, the chicken sera failed to show the variations commonly encountered in similar tests with ferret antisera(8). Ancillary indications for reproducibility are revealed by the expected antigenic homogeneity of virus strains isolated from the same outbreak (*e.g.*, the HSC viruses and the French strains Ba, Nou, Chantal and Fa—Table III) and by the overall similarity of the A prime viruses (Table II) isolated during 1950 and 1951. It should also be pointed out in this connection that reasonable agreement with the results reported in this study can be expected elsewhere only if the strains employed are of common origin to those used here, since it has been repeatedly shown(20,21) that antigenic variation may result from repeated passage in animals or embryonated eggs.

The antigenic formula provides definitive information regarding the reaction of certain antigens of the strain in question with antibodies against the particular antigenic components represented in the prototype antisera. The similarity of a given virus to a prototype strain as revealed by tests with prototype antiserum alone may not be shown when antiserum to the virus in question is tested with prototype antigen. Thus, the Syr II virus (Table III) reacted strongly with WS antiserum in contrast to the weak reaction of WS antigen with Syr II serum. Similarly (Table II) the FM1 virus reacted strongly with FW-1-50 serum while the FM1 serum was essentially devoid of antibody to the latter.

The prototype strains included in the present system for antigenic analysis sufficed to establish formulae for all influenza strains examined in this laboratory to the present time; further experience may indicate the need for including additional prototypes. According to the present system, such additional strains should be designated e, f, g, etc., for the A type viruses and a similar extension could be made for the B and C type viruses. The use of 4 designations (*i.e.*, 0, 1, 2 and 3) for expressing the degree of reaction of virus antigen with prototype antiserum is to be regarded as tentative. The 4 symbols sufficed, in the present study, to show the apparent antigenic changes which are taking place in the A prime group(1,2) and which may be of sufficient magnitude to be of significance with respect to prophylactic immunization. Use of a smaller number of designations (each representing a broader range of antibody titer) in the formulae tended to magnify the 2-fold dilution error of the test while a larger number of designations emphasized the less significant differences between strains. The expediency of the present system will be revealed in further studies along these lines.

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20. Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 143.

21. Hirst, G. K., *J. Exp. Med.*, 1947, v86, 357.

The antigenic relationships shown in the present and previous(1,2) studies are in general agreement with the results of recent investigations by other workers, insofar as comparisons can be made. Thus, van der Veen and Mulder(22) found the A viruses isolated between 1934 and 1943 to be distinct from the A prime group (1946-1949) with few exceptions, and the WS agents, which were clearly separated from the A prime strains, were reasonably distinct from such A type viruses as PR8. Andrewes and Isaacs(16) recently reported the occurrence of 2 antigenic types of influenza A prime virus among 74 strains isolated during 1950-51; these were differentiated by the agglutination-inhibition test with ferret antisera treated to destroy normal inhibitor. Strains designated S reacted in general to very low titer with all A prime ferret sera, even homologous ones, and the L viruses reacted to high titer with homologous ferret sera and much less with other A prime sera. A few strains with intermediate properties were designated SL. The 1950-51 strains we examined (only 5 of our viruses could have been the same as those of the above study) did not show such differences; in contrast, our strains were remarkably homogeneous in spite of the high level of strain specificity which chicken antisera exhibit. The marked antigenic difference between Lee virus and most of the influenza B strains isolated since 1945 was likewise shown in a report by Tamm and associates(23). The present studies confirmed the antigenic identity of 1233 and JJ viruses reported by Francis and associates(6).

Summary. A method for designating antigenic components of influenza A, B and C viruses consisting of formulae derived from the results of agglutination-inhibition tests with chicken antisera prepared against a selected group of prototype strains was presented and discussed. Studies to determine the antigenic patterns of some recently isolated influenza A viruses by the agglutination-inhibition technic revealed that the A prime strains isolated in 1950-51 formed a remarkably homogeneous group resembling the 1950 FW-1-50 (Cuppett) agent. The Benson virus, which was an exception, was indistinguishable from the 1947 FM1 strain. The results of the antigenic analyses of some WS- and PR8-like viruses isolated since 1947 (during which time the A prime form of influenza A was predominant) were also included and their significance discussed.

The technical assistance of Captain E. L. Buescher, M.C., and S.F.C. W. O. Blair is gratefully acknowledged.

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Effect of Hypoglycemia on Rats with Intrasplenic Adrenal Transplants.* (19024)

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Previous reports(1-3) have shown that auto- and homotransplants of the adrenal gland to the spleen of adrenalectomized rats, maintained life, maturation and gonadal function in the recipients. It was suggested that the liver did not destroy the adrenal

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2. Bernstein, D. E., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 175.

3. Bernstein, D. E., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 847.

TABLE I. Effect of Intraperitoneal Injection of 250/kg Alloxan on Rats, with Intrasplenic Adrenal Transplants (Group A), Intrarenal Adrenal Transplants (Group B), Bilateral Adrenalectomy (Group C), and Unilateral Adrenalectomy (Group D).

		Diabetes		Time of death	
Rat No.	Fasting[*] blood sugar	Time after alloxan (hr)	Blood sugar	Hr	Blood sugar
Group A					
1	55	24	153	36–48	
2	51	6	160	36–48	
3	51	24	134	36–48	
4	55	24	294	36–48	
5	54	24	343	36–48	
Group B					
1	55	24	426	50 (sacrificed)	
2[†]	65	6	162	24 (killed by heart puncture)	
3[†]	55	[‡]	[‡]	36–48	
4[†]	49	6	252	36–48	
5	49	6	130	36–48	
Group C					
1	41			2	3
2	46			2	3
3	44			2	0
4	34			2	7
5	49			2	5
Group D					
1	45	24	290	48–72	
2	55	24	176	24 (killed by heart puncture)	
3	41	24	244	Alive at 96 hr	
4	43	24	280	48–72	
5	32	24	200	48–72	

[*] All animals were fasted 16 hr before inj of alloxan. Blood sugar values are expressed in mg %.

[†] At 24 hr Rats 2, 3, and 4 of Group B had blood sugar values of 31, 29, and 17 mg respectively.

[‡] Readings not obtainable.

secretions responsible for the maintenance of these activities.

The purpose of the present investigation is to present evidence that the adrenal secretions which influence sugar metabolism, are likewise not demonstrably affected by passage through the portal circulation.

Method. Twenty mature Long-Evans rats of approximately the same age were divided into 4 equal groups. Group A had been adrenalectomized and had one adrenal autotransplanted to the spleen about 5 months before the experiment. Group B had been adrenalectomized and had one adrenal autotransplanted to the kidney approximately 4 months before the experiment. Group C was adrenalectomized 3 days before the experiment and maintained on saline instead of tap water. Group D had only one adrenal removed 3 days before the experiment. All animals were maintained on a regular diet except Group C as indicated, until 16 hours before the experiment, at which time all food was removed. Fasting blood sugars[†] were taken before the intraperitoneal injection of 250 mg of alloxan per kg of rat. Blood sugar determinations were made at various intervals during the course of the subsequent 48 hours, and autopsies were performed on Groups A and B.

Results. All of the bilaterally adrenalectomized animals (Group C) died within 2 hours after alloxan injection from hypoglycemic convulsions, during which they had exceedingly low blood sugar values (Table I). None of the remaining animals died within 36 hours except 2 that were killed inadvertently by heart puncture at the end of 24 hours. Four animals of Group B showed definite diabetic blood sugar levels within 24 hours, but after this time, 3 of the 4 animals changed to hypoglycemic levels. Their actual death was not observed but occurred 12 to 24 hours after these low sugar level values were attained.

[†] Micro method of Kingsley, J. R., and Reinhold, J. J., *J. Lab. and Clin. Med.*, 1949, v34, 713.

The autopsies on the animals of Group A revealed that all but Rat No. 1 of Group A had large adrenal transplants in the spleen which averaged 4 mm in diameter, and there were no adhesions that would permit circumvention of the portal circulation. This animal showed no evidence of a transplant, however, a 2.5 adrenal accessory was found.

The autopsies on the animals of Group B revealed that 4 out of 5 showed large transplants that varied from 3 to 5 mm in diameter. The fifth animal (No. 2) had a smaller transplant measuring 1 x 2 mm, no accessory adrenal tissue was found. On histologic examination the adrenals that were transplanted to the kidney, unlike those in the spleen, showed some clearly defined medullary tissue in addition to the usual cortical cells.

Discussion. When alloxan has been administered to a normal rat, preceding the establishment of the diabetic state, there is an immediate rise in blood sugar followed by severe hypoglycemia. The latter state is presumed to be due to release of insulin from damaged islets of Langerhans(4-6). Kirschbaum *et al.* (7) reported that after the administration of alloxan to adrenalectomized rats, they all showed hypoglycemic convulsions within 6 hours with the dosage employed, whereas convulsions did not appear in a control, intact group. This reaction was not influenced by absence of the adrenal medulla but was dependent on the adrenal cortex.

In the present experiment, alloxan was employed to induce hypoglycemia. The 5 adrenalectomized animals (Group C) died in 2 hours with convulsions from marked hypoglycemia, as expected from Kirschbaum's report. The control Group D, with one adrenal removed and one left *in situ,* behaved identically as the group with an adrenal in the portal circulation (Group A). The second control (Group B) with an adrenal transplanted to the peripheral circulation, *i.e.*, the kidney, also behaved like intact animals. Since none of these animals died from hypoglycemia, as did the adrenalectomized group, it is suggested that the secretions from the adrenal cortex concerned in carbohydrate metabolism that are elaborated by the intrasplenic adrenal transplant, are not destroyed by passage through the liver. However, since the site of action of cortical hormones is unknown, it can not be stated with certainty that the adrenal protective mechanism against hypoglycemia is not mediated in the liver *per se*.

There is indirect evidence that animals bearing a transplanted adrenal gland do not tolerate stress as well as animals with adrenal tissue *in situ*. The animals of Group D definitely survived longer than Groups A and B when subjected to the same stresses of starvation and diabetes.

Summary. The administration of alloxan to the adrenalectomized animals resulted in hypoglycemic convulsions and death in 2 hours. This did not occur in animals with an adrenal transplanted to the portal circulation, to the peripheral circulation, or when one adrenal gland was removed and the other left *in situ*. Thus indirect evidence is presented that the liver does not destroy the secretions of the adrenal cortex that may be concerned with carbohydrate metabolism.

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5. Ridout, J. H., Ham, A. W., and Wrenshall, G. A., *Science*, 1944, v100, 57.

6. Goldner, M. G., and Gomori, G., *Endocrinol.*, 1944, v35, 241.

7. Kirschbaum, A., Wells, L. J., and Molander, D., PROC. SOC. EXP. BIOL. AND MED., 1945, 558, 294.

A New Blood Factor, s, Allelic to S. (19025)

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On testing the serum of a mother who delivered an infant suffering with hemolytic disease, atypical reactions were observed which did not conform in specificity with any of the known antibodies. The mother, Mrs. Audrey Guth, para 2, gravida 3, had a normal full-term infant, a subsequent abortion at 2 months of pregnancy and on October 27, 1950 a delivery of a severely affected infant, who recovered following 2 transfusions of Rh negative blood.* Preliminary tests showed the father and the affected infant to be in group O, Rh negative (cde/cde), and the mother Rh positive, type Rh_2 (cDE/cde). Titration of the mother's serum on the husband's cells showed the presence of very weak saline agglutinins and incomplete antibodies, demonstrable at about 1:8 with the indirect Coombs test and in tests with trypsinized cells.

Additional studies could not be carried out until April 1951, when the child was 4 months old and free from symptoms. More complete determination of the antigenic components of the red cells of the 4 members of the family are given in Table I.

The mating was incompatible for N, Kidd(1), and more significantly for the factor demonstrable with the antibody in the mother's serum. The antibody 4 months post partum gave weak or negative reactions with saline suspended cells, and its titer in the indirect Coombs was about 1:4. Parallel tests with a panel of selected red cells failed to reveal any parallelism in the reactions of anti-N, anti-Kidd, and the antibody in the Guth serum which gave about 88% positive reactions. These preliminary studies indicated the existence of a new blood factor which induced transplacental isoimmunization and hemolytic disease.

A clue to the specificity of the antibody was revealed on testing the antigenic constitution of all bloods lacking this new blood factor. Remarkably enough, all these bloods gave strong and distinct reactions with anti-S.† In short, on testing numerous bloods with the Guth serum and anti-S, only 3 types of reactions were observed as in the case of M-N, C-c, E-e, and K-k systems. A type of blood lacking both S and the new factor was never observed in tests of more than 800 bloods. On the basis of preliminary findings, the factor was called "s" and its antibody "anti-s."

Since the titer of anti-s 4 months postpartum was low, 3 intravenous injections of 1-2 cc of the husband's blood were administered, as a result of which the titer was increased to 1:32-1:64 in the indirect Coombs test. The titer with saline suspended cells, which was 1:32-1:64 after 2 injections dropped after the third injection to a value of 1:4-1:8 on homozygous bloods ss.

With this more potent anti-s at hand, tests were carried out in parallel with it and anti-S on 353 random white and 145 random colored

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* For the specimens in this case the authors are indebted to Drs. W. R. Platt, pathologist, and R. A. Warwick, pediatrician, at the West Jersey Hosp., Camden, N. J.

1. Allen, F. H., Diamond, L. K., and Niedziela, B., *Nature*, 1951, v167, 482.

† The S factor was discovered in 1947 by the Australian workers Walsh and Montgomery with the aid of an antibody present along with anti-Rh in the serum of a mother of an erythroblastic infant(2). Its relationship to the MN system was described by Sanger and Race in a study of the Australian serum(3). Several examples of anti-S were described either as naturally occurring antibodies or as immune antibodies following a transfusion. Anti-S occurring by itself as the cause of hemolytic disease has as yet not been observed (4).

2. Walsh, R. J., and Montgomery, C., *Nature*, 1947, v160, 504.

3. Sanger, R., and Race, R. R., *Nature*, 1947, v160, 505.

4. Levine, P., *Trans. New York Acad. Sciences*, 1951, v13, 205.

TABLE I. Bloods of the Guth Family Studied with 15 Different Sera.

			D	C	E	c	e	K	k	Duffy	Kidd	S	Mrs. Guth
Father	O	N	0	0	0	+	+	0	+	0	+	0	+
Mother	O	M	+	0	+	+	+	0	+	0	0	+	0
1st child	O	MN	+	0	+	+	+	0	+	0	+	+	+
Affected infant	O	MN	0	0	0	+	+	0	+	0	*	+	+

* Not tested.

TABLE II.

Anti-S	Anti-s	White (%)	Colored (%)	Genotype
+	0	12.18	2.76	*SS*
+	+	46.46	28.96	*Ss*
0	+	41.36	68.28	*ss*
No. studied		(353)	(145)	

individuals. The incidence of the three types of bloods in the 2 groups is given in Table II.

The genetic relationship is further revealed by the gene frequencies, the calculation of which is given below:

Frequency of gene			White	Colored
S	=	$\sqrt{SS}$	.349	.166
s	=	$\sqrt{ss}$	.643	.827
			.992	.993

The values for anti-S in the colored population, *i.e.* 31.72% (SS plus Ss), are in good agreement with that observed by Miller, Rosenfield, and Vogel(5) in a sample of 580 colored individuals *i.e.* 29.5%.

The values of the gene frequencies for the white population in the United States do not differ much from those obtained by Race and his co-workers(6) in their studies on the distribution of only one of these factors, S, in an English population.

Frequency of gene	Race, *et al.*	Present study
S	.337	.349
s	.663	.643

The genetic relationship is further revealed in the so-called double-dose effect of antigenic factors shown by the direct reaction of anti-s both after the second injection and after the third injection when the direct saline titer was considerably lower. With the latter specimen, almost all homozygous bloods were selected by direct reactions with saline suspended cells while distinct reactions for heterozygous bloods could be elicited only after application of the indirect Coombs test.‡

Prior to the discovery of the s factor, it was not clear whether the genetic relationship of the S factor to the MN systems is that of multiple alleles at one locus or of close linkage. The existence of the s factor would seem to indicate linkage as in the case of the CDE/cde factors rather than multiple alleles. Additional evidence to support this view will be published by Sanger and Race(7) to whom a supply of anti-s was made available for testing some of the 123 families included in their genetic study of the factors M, N, and S.

It is hoped that sufficient supplies of potent samples of both anti-S and anti-s will soon become available for comprehensive family and racial studies. As predicted by Fisher (8), the MNSs system is more efficient than either the ABO or Rh-Hr systems for the differentiation of human blood and for rendering decisions of non-paternity. Incidentally, it may be mentioned that the notation of the genes *M* and *N* will eventually have to be altered to make them conform with genetic usage.

Summary. A new blood factor, s, is de-

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5. Miller, E. B., Rosenfield, R. E., and Vogel, P., *Am. J. Phys. Anthrop.*, in press.

6. Race, R. R., Sanger, R., Lawler, S. D., and Bertinshaw, D., *Heredity*, 1949, v3 part 2, 205.

‡ The double dose effect for direct selection of individuals homozygous for S could not be demonstrated with anti-S used in these studies. For a supply of this serum, the authors are indebted to both Drs. Vogel and Rosenfield and to Dr. Mourant.

7. Sanger, R., and Race, R. R., in preparation.

8. Fisher, R. A., cited by Race *et al.*(6).

scribed with the aid of an antibody produced by transplacental isoimmunization. Evidence is presented to show its genetic relationship to the S factor, which is probably linked to the M and N system.

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Quantitative Biologic Activity of $\Delta^{4,6}$ dehydrocortisone as Compared to Cortisone.* (19026)

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The compound $\Delta^{4,6}$dehydrocortisone acetate was first recognized as a byproduct of the partial synthesis of cortisone acetate(1). It is commonly designated as the "diene" of cortisone. The diene is important from the standpoint of establishing correlations between the chemical structure of steroids and their biologic and therapeutic effects. In a preliminary report, Kendall(1) noted that the diene did not suppress the symptoms of rheumatoid arthritis, although its glycogenic potency seemed to equal that of cortisone. Some apparent differences in the physiologic properties of the two compounds were reported by Higgins, Woods, and Kendall(2).

In the present study we have compared the potency of cortisone acetate and the diene acetate in the muscle-work test of Ingle(3) and in the glycogen deposition test. According to these criteria the quantitative potency of the diene acetate is probably no greater than 50% of the potency of cortisone acetate.

Methods. Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they reached a weight of 200 ± 2 g. The procedures used for the stimulation of muscle were according to Ingle(3). The animals were anesthetized with phenobarbital sodium and cyclopal sodium and were subjected to the work test immediately after removal of the adrenal glands and the kidneys. A Nerve Stimulator, Model B, Upjohn, was used to deliver 5 pulses per second. The duration of each pulse was 20 msec. and the intensity was 20 ma. The distance the weight was lifted was recorded on automatic work adders. Each recorder revolution represented approximately 400 g-cm of work. Stimulation was continued until muscular responsiveness was lost. During the work test, the animals were enclosed in a cabinet with the temperature constant at 28° ± 0.5°C. The steroids were dissolved in peanut oil and were given by intermittent subcutaneous injection at the beginning of stimulation and 6, 24, 30, 48, etc. hours later. Each animal received continuous subcutaneous injections of 0.9% sodium chloride solution at the rate of 20 cc per 24 hours per rat. The liver glycogen deposition tests were carried out in the Endocrinology Department of the Upjohn Research Division by Mr. D. F. Kiel. The procedure has been described by Pabst, Sheppard, and Kuizenga(4).

Experiments and results. The data on dosages, numbers of animals and work performance are summarized in Table I. The work response elicited by cortisone acetate

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* We wish to express our appreciation to Dr. E. C. Kendall, The Mayo Foundation, Rochester, Minnesota, who supplied the diene acetate; to Dr. Augustus Gibson, The Medical Division of Merck and Co., who supplied the cortisone acetate; and to Mr. D. F. Kiel, Endocrinology Department, The Upjohn Research Division, who performed the bioassays of these two compounds by the liver glycogen deposition test.

1. Kendall, E. C., *Fed. Proc.*, 1950, v9, 501.
2. Higgins, G. M., Woods, K. A., and Kendall, E. C., *Endocrinol.*, 1951, v48, 175.
3. Ingle, D. J., *Endocrinol.*, 1944, v34, 191.
4. Pabst, M. L., Sheppard, R., and Kuizenga, M. H., *Endocrinol.*, 1947, v41, 55.

TABLE I. Work* Performance of Adrenalectomized-Nephrectomized Rats Given Steroids by Twice-Daily Subcutaneous Injection. Average total recorder revolutions with standard deviations of the averages.

Dose steroid, mg/24 hr/rat	Cortisone acetate		Diene acetate	
	No. rats	Total work	No. rats	Total work
.25	15	17617 ± 877	15	13874 ± 696
.50	14	23697 ± 1703	14	18234 ± 1357
1	19	28205 ± 1433	19	22458 ± 1601

* Each recorder revolution represents approximately 400 g-cm of work.

was significantly greater than the response to the diene acetate at each dosage level. It was necessary to administer amounts of the diene which were twice as great as those of cortisone in order to obtain the same level of work output. Upon this basis it was concluded that the potency of cortisone acetate in the muscle-work test is approximately twice that of the diene acetate.

In the liver glycogen deposition test one unit of activity is defined as the activity equivalent of 0.1 mg of 17-hydroxycorticosterone. The diene acetate assayed 1.86 units per mg and cortisone acetate which was tested in parallel under identical conditions assayed 4.5 units per mg.

Discussion. The results of these experiments show that cortisone acetate is more active than the diene acetate in both the muscle-work test and in the liver glycogen deposition test. The question can be raised as to whether there are any true qualitative differences in the biologic properties of these two compounds or whether the reported differences can be explained on a quantitative basis.

The failure of the diene to suppress the symptoms of rheumatoid arthritis(1) may have been due to insufficient dosage since the only dose tested (100 mg daily) represented the average effective dose of cortisone acetate.

In the studies of Higgins, Woods, and Kendall(2) comparisons of the two compounds were made in normal and in adrenalectomized rats given 2 mg per rat per day. Cortisone acetate suppressed the weight of both non-adrenalectomized and adrenalectomized animals to a much greater extent than did the diene acetate. Cortisone acetate caused a greater regression of the thymus and had a more striking lymphocytolytic effect than did the diene acetate. These differences are explicable by the assumption that cortisone acetate is the more potent of the two compounds and that larger doses of the diene would have caused similar changes. On the other hand, the observations that the diene acetate had a mild lymphocytopoietic effect in normal but not adrenalectomized rats, and that it caused a greater compensatory atrophy of the adrenal cortices than did cortisone acetate may require some other explanation. The question cannot be answered without further comparisons of the biologic properties of the two steroids in which the dose-response relationships are fully explored.

Summary. Cortisone acetate and $\Delta^{4,6}$dehydrocortisone acetate were compared in the muscle-work test and by the liver glycogen deposition test. According to these criteria the quantitative potency of the diene is probably no greater than 50% of the potency of cortisone acetate. It is suggested that the reported differences in the biologic properties of these two compounds may be explicable by the assumption that larger doses of the diene would cause biologic responses which are identical with those caused by cortisone.

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Hormone-Secreting Transplantable Neoplasms of the Pituitary Induced by I^{131}.* (19027)

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Among the abnormal growths those of the endocrines and their target organs are the most difficult to characterize. Are the nodules of the adrenal, thyroid, prostate, and pituitary, which are commonly encountered in man and animals, true tumors or mere hyperplasias produced by endocrine overstimulation or dysfunction? Where is the borderline between hyperplasia and neoplasia, and what are the criteria to distinguish between them? Ovaries implanted into the spleens of castrated rats and mice invariably give rise to granulosa cell masses, by virtue of an endocrine disturbance brought about by destruction of gonadal hormones in the liver and secondary stimulation of the pituitary gland (1). Are these nodules true tumors? Stimulated by excessive gonadotropic hormones the granulosa cells grow progressively, but only a few such growths are transplantable to normal hosts. Occasionally, however, they can give rise to malignant growths(2). Gorbman(3) discovered that destruction of the thyroid by I^{131} gives rise to pituitary growths, presumably by overstimulation of the pituitary due to lack of thyroid hormone (TH). Are these growths true neoplasms, or merely hyperplasias? The answer to this question called for transplantation studies. Similarly long continued administration of antithyroid compounds causes the development of chromophobe tumors of the pituitary gland(4).

In our preliminary experiment we fully confirmed Grobman's observations and all mice receiving thyroid destructive doses of I^{131} and living longer than 13 months after such treatment developed pituitary growths (Table I). Mice of both sexes of the C57 strain were given $NaHSO_3$—NaCl solution subcutaneously in volumes of 0.2 cc and containing 200 to 400 μc of carrier free I^{131}. Immediately after injection many animals were lowered in the 100% geometry γ-ray ionization chamber(5) to determine the precise quantity of radioactivity introduced.

The mice were 6 to 10 weeks old when injected with either I^{131} or tumor fragments. Some were obtained through courtesy of Dr. William L. Russell, others from the Jackson Memorial Laboratory, and still others were raised by us. They were fed a standard laboratory diet including purina chow, given *ad libitum*.

The grafts were made into the leg muscle. The animals were kept until death or were killed only *in extremis*, with the exception of a few mice killed in order to study the histogenesis of pituitary growths and related changes. The head was opened under aseptic conditions, and the induced pituitary growths of 14 mice were removed for transplantation. Microscopic nodular, or ill-defined, tumor-like areas appeared in the pituitary 9 months after the I^{131} treatment, and gross tumors after about 10 months. Most mice living longer than 10 months listed in Table I were killed when a marked, usually sudden weight loss ("pituitary cachexia") and listlessness suggested the presence of a pituitary tumor. In the course of 8 months fragments of pituitary growths from 14 mice were implanted in 140 I^{131} pretreated or normal mice. The results of 9 transplantation experiments, the recipients of which lived longer than 6 months, are summarized in Table II. Some were

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* Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

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2. Furth, J., and Sobel, H., *J. Nat. Cancer Inst.*, 1947, v8, 7; and Li, M. H., and Gardner, W. U., *Cancer Res.*, 1947, v7, 38.

3. Gorbman, A., *J. Clin. Endocrinology*, 1950, v10, 1177.

4. Seifter, J., Ehrich, W. E., and Hudyma, G. M., *Arch. Path.*, 1949, v48, 536.

5. Atomic Energy Commission Declassified Document No. 2637. Office of Technical Services, Department of Commerce, Washington, D.C.

TABLE I. Induction of Pituitary Growth by I^{131}.

Survival time after inj., mo.	No. in group	Result of treatment: Negative	±*	Pretumor	Tumor
>3	19	19			
3– 8½	31	31			
9– 9¾	4	1		3	
10–11¾	10	2	1	2	5
12–12¾	9	1	1		7
13–13¾	8				8
14–14¾	5		1		4
15–15¾	7				7
16–18¼	6				6

* The presence of a pretumor or tumor in these was probable but could not be fully established.

TABLE II. Summary of Transplantations of Nine I^{131} Induced Pituitary Growths.

		Recipients: I^{131} pretreated No. grafted	No. +	Normals No. grafted	No. +
Original growth	♀	18	10	59	0
	♂			20	0
Subpassage	♀	9	9	8	0

grafted on I^{131} pretreated or normal mice only, some were grafted on both.

All 3 primary pituitary growths which were grafted in I^{131} pretreated mice proved transplantable, while none of 7 similar (or the same) growths grew in normal mice. The first subpassage attempted succeeded in all 9 I^{131} pretreated mice and in none of the 7 normal mice of the same strain, age and sex. The I^{131} treatment was administered about 3 to 5 weeks prior to the graft so that a sufficient time elapsed for destruction of the thyroid. Indeed, destruction of the thyroid, we believe, created the necessary stimulus for the multiplication of the grafted pituitary cells.

The tumors grafted in the thigh appeared after about 2 to 3 months. They were firm, grey, "healthy" growths with little or no gross necrosis. There was a conspicuous stimulation of uteri in all tumor-bearing female mice. The ovaries of mice bearing grafted tumors invariably assumed the appearance of those receiving gonadal pituitary hormones or urine from pregnant women, while those bearing primary pituitary growths were atrophic and filled with lutein cells, as those of X-rayed mice. This difference is probably due to the age of the hosts; the former being young, the latter old with atrophic ovaries devoid of ova and normal follicles, non-responsive to pituitary gonadotrophins. This finding is indicative of discharge of large quantities of gonadotropins by the grafted tumors.

It can be assumed that thyrotropic hormones (TSH) are also produced by the neoplastic cells, but the very condition that made the growth of these tumors possible (destruction of the thyroid) made it impossible to observe the morphologic evidence of the presence of TSH. Bioassays are in progress. The changes preceding the development of these pituitary tumors will be more fully described. All tumors are chromophobes and appear to be derived from either basophils or chromophobes.

Comments. These transplantation experiments indicate that maintenance of growth of these pituitary tumors depends on destruction of the thyroid gland and lack of TH. If this is true, injection of TH will prevent the development of pituitary tumors. Patients with thyroid carcinoma heavily treated with I^{131} usually receive thyroid hormones when the signs and symptoms of myxedema are advanced. Therefore, patients so treated are not expected to develop pituitary neoplasms.

The morphologic features of the neoplastic pituitary cells are similar in different animals: all are "chromophobes," and all tumors invariably secrete gonadotropic hormones as indicated by the appearance of uteri and ovaries of tumor-bearing hosts. Presumably, all secrete also TSH and it seems probable that the same cell manufactures both hormones. Accordingly, one would expect that if hyperplasia of these cells is brought about by a gonadal imbalance, the stimulated cells will also discharge thyrotropic hormones in excess. We are not familiar with clinical experience indicating that this is true.

If one cell produces different hormones, how is the specificity of stimulation maintained? If the induction of these pituitary growths is caused by destruction of the thyroid, would thyroidectomy also incite the development of such neoplasms? Will these conditioned neoplasms change into unconditioned neoplasms in the course of transplantation or in some hosts bearing them, as do the hyperplastic granulosa cell mass induced by the Biskind procedure(2)? Neoplastic cells are abnormal cells which frequently have a characteristically larger or smaller concentration of enzymes than their normal precursors. Will a similar dissociation occur with hormone secreting cells when they turn neoplastic? On basis of experience with liver tumors, it can be anticipated that some pituitary tumors may discharge one hormone only. Could such a dissociation be induced, or a selective depression of secretion of one hormone be brought about? It is possible, though not likely, that hosts whose thyroid had been destroyed by I^{131} are also susceptible to other neoplasms to which they are normally resistant. There are many different experiments which can be designed to answer these questions. It seems that a follow-up of Gorbman's discovery might yield new basic information concerning both the physiology of the pituitary and control of some neoplasms.

Summary. (1) Pituitary growths induced by thyroid destructive doses of I^{131} are readily transplantable in mice whose thyroid glands had been similarly destroyed, but not in normal mice. (2) These growths discharge gonadotropic hormones which in turn stimulate the production of gonadal hormones. Discharge of thyrotropic hormones by these tumors is assumed but remains to be demonstrated. (3) On the basis of observations made it is postulated that (a) the growths of the pituitary induced by thyroid destroying quantities of I^{131} are conditioned neoplasms formed by cells which are driven to proliferation through the stimulus created by the absence of TH; (b) the same chromophobe pituitary cell can discharge both gonadotropic and thyrotropic hormones.

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Glucose-Utilization in Hepatectomized Dogs.* (19028)

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In experiments here reported the rate of disappearance (or utilization) of sugar from the blood in hepatectomized dogs was studied immediately after operation, by determinations of the blood-sugar levels at intervals before and after the intravenous injection of glucose solution. In eviscerated dogs Soskin and Mirsky(1) observed that the rate of disappearance of blood-sugar, expressed in percentage of initial concentrations, was constant before and after intravenous injections of glucose. Comparing the behavior of eviscerated dogs and hepatectomized dogs, DeDuve(2) estimated the rate of utilization of glucose from amounts of intravenously in-

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† Postdoctorate Research Fellow, 1948-50, National Institute of Health, U. S. Public Health Service.

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2. Duve, C. de, *Compt. Rend. Soc. de Biol.*, 1947, v141, 1145.

TABLE I. Changes of Blood-Sugar in Dogs Immediately After Hepatectomy, Before and After the Intravenous Injection of Glucose. Values in mg of sugar per 100 cc of blood.

Dog No.	Min after hepatectomy					Min after administration of glucose				
	0	15	30	45	60	5*	20	35	50	65
1	67	59	51	44	37	223	170	125	103	85
2	66	55	46	40	33	282	185	156	129	110
3	95	88	76	70	64	274	208	184	163	149
4	71	61	55	50	43	243	173	144	134	123

* *i.e.,* 65 min after hepatectomy.

jected glucose that were required to maintain the blood-sugar level constant. Finding the rate of utilization faster in hepatectomized than in eviscerated dogs, DeDuve suggested that the higher rate in the dogs with simple hepatectomy was related to their ability to produce insulin. This hypothesis is supported by observations in the present experiments.

Procedures. Mongrel male and female dogs were fasted 3 days before operation, with water allowed *ad libitum*. Hepatectomy was performed by the method of Firor and Stinson (3), under nembutal anesthesia. Concentrations of glucose in venous blood were determined in duplicate, by the Nelson-Somogyi method(4), on samples drawn immediately and at 15-minute intervals for one hour following the hepatectomy. Glucose was then administered intravenously, in dosage 0.5 gram per kilogram of body weight, given in 50% solution within a period of 3 minutes, and levels of glucose in the blood were determined at the same intervals for another hour. Urine was collected at the end of each period and tested for glucose, but no glycosuria was found.

Results. Data in Table I, from experiments on four hepatectomized dogs, show the disappearance of sugar from the blood after operation during the first hour, before the injection of glucose, and in the second hour, after glucose solution was administered intravenously. The graphs in Fig. 1 represent averages of the blood-sugar values, expressed in percentage of initial levels, demonstrating a faster rate of fall after the glucose-injections. Statistical examination of the data by the method of analysis of variance(5) shows a highly significant difference ($p < .001$) between the two rates of disappearance of sugar.

Fig. 2 presents, for comparison, averages of the data originally reported by Soskin and Mirsky(1) on changes of blood sugar following evisceration (removal of liver and intestinal tract, with kidneys left *in situ* with ureters ligated) in 6 dogs, before and after the intravenous injection of glucose-solution. The latter graphs show no difference in the rates of disappearance of blood sugar before and after the administration of glucose, despite the fact that the elevated blood-sugar levels produced by injections of glucose were closely comparable to the levels produced in our experiments.

Discussion. Differences in rates of disappearance of blood-sugar before and after the administration of glucose in these hepatectomized dogs, in contrast with the absence of such differences in eviscerated animals as reported by Soskin and Mirsky(1), may be reasonably ascribed to the presence of the pancreas and its potential response. It seems probable that the intravenous injection of glucose in the animals with intact pancreases may stimulate the secretion of insulin and thus accelerate the rate of disappearance of sugar from the blood—an effect not seen in eviscerated animals from which the pancreas was removed.

Summary. In 4 *hepatectomized* dogs the rate of disappearance of glucose from the blood during the first hour after operation was slower than during a second hour, after the intravenous injection of glucose solution. This is in contrast to previously reported findings by others that in completely eviscerated dogs there is no difference in rates of dis-

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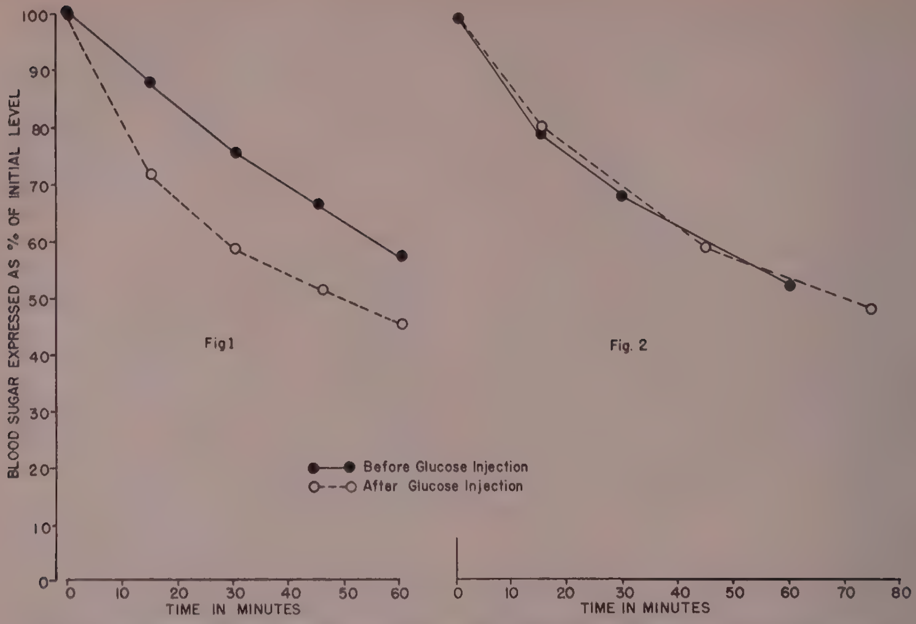

FIG. 1. Averages of blood sugar levels in 4 hepatectomized dogs before and after intravenous administration of glucose.

FIG. 2. Averages of blood sugar levels in 6 eviscerated dogs, before and after intravenous administration of glucose (experiments of Soskin and Mirsky) (1).

appearance of blood-sugar before and after the administration of glucose. The findings suggest that in hepatectomized animals the intravenous injection of glucose stimulates the secretion of insulin which accelerates the rate of removal of sugar from the blood by tissues other than liver.

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Effect of Early Small Exchange Transfusion on the X-Irradiated Dog.* (19029)

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During the course of a series of experiments on the hemolytic action of ionizing radiation, utilizing the technic of differential agglutination of erythrocytes in the dog, 4 dogs were exposed to 450 r whole body X-irradiation. Within ½-hour after completion of irradiation the dogs were rapidly bled of 250 cc and transfused with an equal volume of fresh, normal, compatible and serologically identifiable dog blood(1). The results of the studies on the fate of the transfused erythrocytes will be reported elsewhere. Although the dosage of X-irradiation given these dogs

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* This study was aided partially by a contract between The University of Rochester and The Office of Naval Research, Department of the Navy (NR 131-174) and partially under a contract with The United States Atomic Energy Commission at The University of Rochester Atomic Energy Project.

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is approximately an LD 70 in this laboratory, none of the 4 animals in the above group died or became ill with the radiation syndrome. The following experiment was designed to determine if this procedure of small exchange transfusion immediately after irradiation had any therapeutic effect on the irradiated dog.

Methods. Twenty healthy dogs of the mixed beagle and mongrel breed weighing approximately 20 lb used in this laboratory for irradiation studies were paired for sex and weight into two groups. The animals had been immunized for distemper and had been given a vermifuge approximately 6 weeks before use. They were kept in separate cages and fed a diet of Purina dog chow with free access to water both before and during the experiment. Control determinations of hematocrit, white blood cell count, and platelet count were made on blood obtained by jugular puncture on each of 3 days of the week prior to irradiation. Standard laboratory methods were used in all of these determinations. The animals to be transfused were typed for presence of the canine "A" factor in their erythrocytes as described by Christian, *et al.*(2) and Young, *et al.*(3). Compatible donors were then chosen from a donor colony of healthy large animals, giving A+ dogs either A+ or A– blood, but only A– blood to A– recipients. Cross matches of donor's cells and recipient's serum, and recipient's cells and donor's serum were carried out in small tubes utilizing fresh plasma, and cells suspended in fresh plasma incubated together for 15 minutes at room temperature followed by centrifugation, as an additional precaution against hemolytic transfusion reactions. The pairs of animals were then exposed to 550 r delivered from a 1000 KVP General Electric Industrial X-ray unit operated at 3 ma using a lead plano-convex filter, ½-inch thick in the center. Radiation was delivered at a rate of 6.4 r/minute as measured by a Victoreen chamber prior to exposure of each pair of animals. Target to skin distance was 38 inches. Animals were exposed in a standing position while confined to the uniform field area in a large mesh wire cage. Within 30 minutes of completion of irradiation, 250 cc of blood were removed during a 5- to 10-minute period from the animal chosen for transfusion, by jugular puncture. Immediately after bleeding, the animal was given 250 cc of whole donor blood by rapid drip into a leg vein during a 10- to 15-minute period. Donor blood was dawn in 250 cc units from 1 to 4 hours before use into 40 cc of cold acid citrate dextrose blood preservative (ACD) solution in standard Baxter vacuum bottles and stored at 4°C until used. A mild shock-like picture after bleeding was seen in 2 of the 10 animals before transfusion was instituted. This was rapidly controlled by transfusion. In 2 of the animals, mild immediate reactions to transfusion occurred. These were characterized by restlessness, hyperpnea, some instability of gait, and vomiting or retching. These findings disappeared within 15 minutes. Post-transfusion serum samples were free of hemolysis, and hemoglobinuria was not seen. In our opinion, these reactions were not hemolytic in nature but were a manifestation of inability of the animal to readjust his cardiovascular system to the rapid changes in blood volume. From other experiments carried out in a similar manner on dogs of this size while transfusing serologically identifiable cells(1), it has been shown that approximately 15 to 20% of the cells in the immediate post-transfusion blood specimens are donor cells. Thus it can be assumed that approximately that fraction of the recipient's blood volume was replaced with donor blood in the present experiment. Controls were untreated. Following irradiation, peripheral blood was obtained from both control and transfused animals either several hours after irradiation or on the day following irradiation and then at 3- or 4-day intervals for the same determinations that were obtained during the pre-irradiation period. Animals in both groups were given only standard kennel care and subsequent transfusions were not administered. Clinical observations were recorded daily on all animals. Animals

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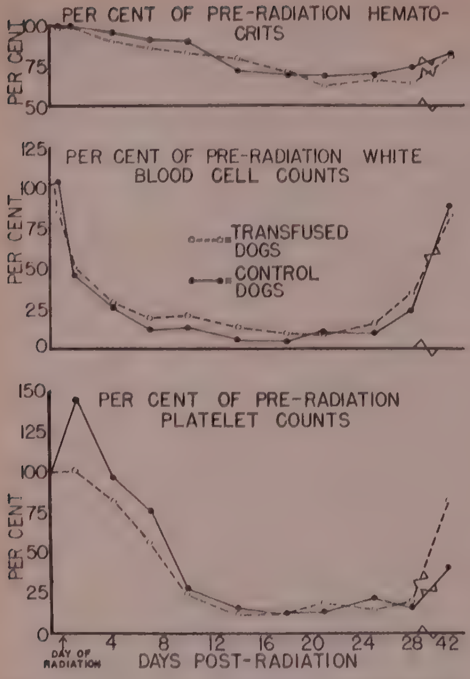

FIG. 1. Graph of average change in hematocrits, white blood cell counts, and platelet counts in control and transfused groups of animals. Three pre irradiation determinations on each animal, were averaged for 100% value for that animal.

that died were autopsied immediately.

Results. Fig. 1 is a graph of the changes in average white cell count, platelet count and hematocrit in the two groups. The 3 pre-irradiation values for each of these determinations were averaged for each animal and were considered as the 100% value for that animal. Subsequent changes were calculated as per cent of this initial value. Fig. 1 shows the average of these percentages for the two groups. It will be seen that there is no significant difference in white cell count or hematocrit between the transfused and control groups. Platelet counts were made on only four pairs of animals on the first post-irradiation day. The difference in platelet counts observed has been tested statistically and a non-significant "t" obtained. Similarly, the difference in platelet counts observed 6 weeks post-irradiation is not significant because of the small number of survivors. Clinical observations revealed no difference between the groups. Survivors in both groups remained clinically well or became only mildly ill except for one surviving transfused animal who developed bilateral retrobulbar hemorrhage and necrosis of the orbits. Although this animal survived, he was sacrificed 45 days post-irradiation because of this lesion. Autopsy revealed no lesion which might be expected to cause death. Animals in both groups who died became clinically ill 3 to 4 days before death.

Fig. 2 shows the cumulative mortality experience, indicating the total number of animals in each group dead on each post-irradiation day. Total mortality in the control group was 70%, and was 40% in the transfused group at the end of 45 days. Calculation of χ^2 indicates that there is a 20% chance that a difference as great as this could be observed if in fact there were no differences in the two groups. This is a rather low level of reliability and the final significance of the observed difference could only be settled by an experiment involving larger numbers of animals. Survivors were randomly distributed among the pairs of animals irradiated together. The marked delay of 7 days in onset of morbidity and mortality in the transfused group would seem to be of much more significance. Six of the seven control dogs who died had succumbed 3 days before the first of the transfused animals died. Previous experience in this laboratory with similar doses of irradiation in similar animals shows that in all groups of control animals the mortality was largely complete by the 21st day, post-irradiation, and in no instance was a delay in onset of mortality to the 21st day post-irradiation encountered. This difference in onset of mortality in the two groups leads

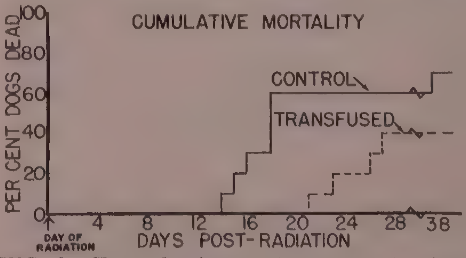

FIG. 2. Chart showing total number of animals dead on each post-irradiation day.

us to feel that the observed difference in ultimate mortality may well be significant.

Autopsy disclosed no significant difference in cause of death between the dead animals of the two groups. All deaths were principally due to widespread hemorrhage into all organs. Similarly, clinical examination of the survivors of the 2 groups at the end of the experiment revealed no differences of note.

Discussion. It has recently been shown that mortality from ionizing radiation exposure can be influenced in a number of ways in various species. Jacobson has shown that shielding of the spleen during irradiation(4) or surgical transfer of normal splenic tissue immediately post-irradiation(5) significantly increases the tolerance of mice to X-irradiation. Allen has observed a similar effect in dogs when the head is shielded during irradiation (6). Salisbury and colleagues demonstrated a reduction in mortality in a small number of irradiated dogs effected by a single massive cross transfusion carried out in the immediate post-irradiation period(7). There was no apparent delay of onset of mortality in their group of cross transfused animals. Brecher and Cronkite have observed a decrease in mortality in rats in whom parabiosis was established after irradiation(8). These observations and the data reported here would seem to be in accord with the hypothesis that early transfer of some cellular or humoral factor from a normal to an irradiated animal by a variety of means or protecting tissue which may contain such a factor during radiation exposure can beneficially influence the subsequent course of the animal's response to radiation injury. Studies thus far reported give little indication of the nature of such a factor or of its mode of action. It may be hypothesized that this factor acts (1) to hasten the onset of repair processes; (2) to inhibit or remove some toxic product produced by radiation; (3) to minimize the ultimate cell damage initiated by irradiation; or (4) to transplant blood forming cells directly from donor to recipient(8). The data of Jacobson showing a rapid return of marrow activity in spleen-protected mice indicate that the effect may be to hasten onset of repair processes. The data reported here suggest that the hypothetical factor concerned may be present in whole blood. The present experiment does not separate the possible beneficial effect of bleeding and subsequent transfusion, or of the effect of ACD solution received by the transfused animals. The lability of such an hypothetical factor during storage of the blood is likewise not considered. Thus, interpretation of the mechanisms by which the observed results occurred must be made with these limitations in mind.

The findings in the present experiment suggest the feasibility of attempting immediately after exposure relatively large exchange transfusion of fresh blood in humans exposed to doses of whole body irradiation in the range of high lethality such as might occur with an accidental laboratory exposure. It is probable, however, that with doses appreciably above the LD 100 level ultimate mortality would not be affected.

Summary. 1. Immediate removal of 250 cc of blood and replacement with an equal volume of normal compatible whole blood in 20 lb dogs given 550 r whole body irradiation (expected LD 80) results in a marked delay in onset of radiation syndrome and mortality. There is a suggestive decrease in ultimate mortality as well. 2. No differences in average leucocyte count, platelet count, or hematocrit were observed in the two groups, nor were there differences in clinical or postmortem anatomical findings. 3. The implications of these results are discussed.

The authors are indebted to Drs. Joe Howland and L. E. Young for advice and facilities provided for these studies, and to Dr. Arthur M. Dutton for statistical aid.

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Methylation of Nicotinamide in Patients with Pernicious Anemia. (19030)

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(Introduced by C. C. Sturgis.)

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There is increasing evidence in experimental animals that both vit. B_{12} and folic acid are involved in methylation processes (1-3). It appeared possible therefore that the pernicious anemia patient, who according to clinical evidence is deficient in both folic acid and vit. B_{12}(4), would lack the ability to methylate certain compounds and excrete their methyl derivatives. Furthermore, it was considered that the degree of methylation of these compounds might constitute a sensitive measure of the nutritional adequacy of vit. B_{12} and folic acid. Nicotinamide has been shown by several investigators(5,6) to be excreted in the urine of man and experimental animals in part as N^1-methylnicotinamide. Ellinger and Hardwick(8) gave some evidence that in man the level of N^1-methylnicotinamide excreted in the urine might be affected by exhaustion of "methyl donators."

In the present experiment, nicotinamide was administered for 3 consecutive days in doses of 500 or 1500 mg to 4 patients with pernicious anemia before and after therapy was instituted with either vit. B_{12} or folic acid. The urinary excretion of N^1-methylnicotinamide was measured for each 24-hour period according to the fluorometric procedure of Huff and Perlzweig(9). The N^1-methylnicotinamide used as a standard was obtained from the Delta Chemical Works. The results are recorded in Table I.

TABLE I. Methylation of Nicotinamide in Pernicious Anemia Patients.

		N^1-methylnicotinamide excreted	
Patient	Dose of nicotinamide, mg	Before R_x, mg/24 hr	After R_x, mg/24 hr
A	—	16	4.1
	—	13.4	5.6
	500	50	93.3
	500	114.8	91.5
	500	132.2	122.1
% of total dose of nicotinamide		18	18
R	—	7	2.3
	—	8.4	2.7
	1500	414	417
	1500	495	201.7
	1500	651	278
% of total dose of nicotinamide		31	18
We	—	3.4	2.6
	—	3.8	2.1
	1500	190	180.5
	1500	298	286
	1500	423	324.5
% of total dose of nicotinamide		18	16
Wa	—	14.9	4.8
	—	6.4	5.6
	1500	217.6	198.5
	1500	324.1	312.2
% of total dose of nicotinamide		15	15

Under the conditions of the experiment, in all 4 patients a significant amount of nicotinamide, from 15 to 31% of the total administered dose, was excreted in the methylated form. No differences in amount of the methylated derivative were obtained before therapy and after remission had been induced

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with either vit. B_{12} or folic acid administration. In all cases, the values for the N^1-methylnicotinamide tended to increase on the third day of nicotinamide administration. Hence there is no apparent trend toward exhaustion of "methyl donator stores."

The values for the 24-hour excretion of N^1-methylnicotinamide previous to nicotinamide supplementation as recorded in Table I are all within the range of 3 to 17 mg reported by Huff and Perlzweig(10) for 67 normal male medical students. The values of methylated compound excreted are somewhat higher in terms of percent of nicotinamide administered than other reports(9) in the literature, but the nicotinamide dose in these experiments was also greater.

There is no indication that under the conditions here employed the methylation of nicotinamide can be used as a measure of the nutritional adequacy of vit. B_{12} or folic acid. Apparently the state of vit. B_{12} and folic acid deficiency existing in pernicious anemia does not influence the patient's ability to methylate nicotinamide.

It is possible that the administered doses of nicotinamide are not large enough to produce a condition of metabolic stress on the "methyl donators." It is also considered possible that in humans, vit. B_{12} and folic acid are not concerned in the enzyme systems governing either the transfer or formation of the methyl group of N^1-methylnicotinamide.

Summary. Administration of nicotinamide to four patients with pernicious anemia results in the urinary excretion of approximately 20% of the compound as the methylated derivative. The levels of N^1-methylnicotinamide are similar before and after therapy. The methylation of nicotinamide, under the conditions here employed, cannot be used as an index of the nutritional adequacy of vit. B_{12} or folic acid.

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Influence of Desoxycorticosterone Acetate and Cortisone Acetate on Body Weight of Chick Embryos.* (19031)

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A retardation in growth of chick embryos without increasing the incidence of developmental defects was noted by Landauer(1) following the injection of adrenal cortical extract into the yolk sac. Recently, Karnofsky and his associates(2,3) have studied cortisone acetate and noted the effects of this steroid on chick embryo development. A definite growth inhibiting action was demonstrated for cortisone acetate and the "cortisone-effect" also involved characteristic developmental modifications. It appeared of interest to estimate the effect of an adrenal steroid not oxygenated at C-11 and for this purpose desoxycorticosterone acetate (DCA) was chosen for comparison with cortisone acetate at the same dosages.

Varying amounts of DCA (Percorten, Ciba)† and cortisone acetate (Merck) were injected into the yolk sac of White Leghorn chick embryos on the 6th day of incubation. The embryos were sacrificed on day 18,

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† Grateful acknowledgement is made to Drs. P. Sturkie and J. Pino of the Agricultural Experiment Station for the eggs and to Ciba Pharmaceutical Products for the desoxycorticosterone acetate (Percorten).

TABLE I. Influence of Adrenal Steroids on Body Weight of Chick Embryos Injected on Day 6 and Body Weight Taken on Day 18 of Incubation.

No. embryos	Treatment	Avg body wt, g
17	.25 mg desoxycorticosterone acetate	17.7 ± 1.2*
17	Uninjected controls	24.5 ± .6
30	.50 mg desoxycorticosterone acetate	15.6 ± .9
24	.1 ml sesame oil	20.9 ± .4
30	Uninjected controls	20.9 ± .5
14	.25 mg cortisone acetate	24.5 ± .8
28	.50	20.6 ± .7
14	1.00	20.6 ± 1.1
21	Uninjected controls	24.9 ± .5

$$* \ E = \sqrt{\frac{\Sigma d^2}{n(n-1)}}.$$

weighed and examined for morphological abnormalities. Non-injected or vehicle injected eggs were incubated with each experimental group as some variation in body weight was noted among the control embryos.

The data are presented in Table I. It is evident that DCA when administered as one injection of 0.25 mg effectively retarded body weight increase and that a dosage of 0.5 mg was no more effective. Both dosages, however, significantly retarded body weight increase without inducing abnormalities whereas sesame oil alone had no effect. In comparison, cortisone acetate did not influence body weight increase at the 0.25 mg dosage in contrast to DCA. However, 0.5 mg significantly depressed body weight gain but again a doubling of the steroid dose was no more effective. The dosages of cortisone acetate which were effective on body weight did not induce morphological defects. These data are in essential agreement with Karnofsky, Ridgway, and Patterson(3) who consider the minimum effective dose of cortisone acetate for an 8-day embryo to be 1.5 mg, the LD_{50} being 2.0 mg. These investigators(3) established a minimum dosage effect at a level which caused morphological changes but did find that smaller amounts of cortisone acetate had some effect on body weight only. Karnofsky, Ridgway, and Stock(4) mention that DCA is active at 0.9 mg per egg. In contrast, a significant growth depressing effect was obtained in our studies with 0.25 mg of DCA, a dosage which proved ineffective with cortisone acetate. The mechanism of this steroid action is not apparent.

Summary. A retardation of body weight increase in White Leghorn chick embryos was obtained by injecting either desoxycorticosterone acetate or cortisone acetate into the yolk sac at 6 days of incubation and observing the effects on day 18. Desoxycorticosterone acetate depressed body weight increase at a dosage level of 0.25 mg per egg, whereas 0.5 mg of cortisone acetate was required to induce the same effect.

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Adaptation of Leon Strain of Poliomyelitis to Mice. (19032)

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The increased amount of experimental work in the field of poliomyelitis made possible by Armstrong's successful adaptation of the Lansing strain to rodents is well known(1). Since that time a number of attempts have been made by workers in the poliomyelitis field to repeat this adaptation with other strains. Thus far the only successful results have apparently been obtained with strains which are identical with or immunologically related to the Lansing strain(2-5). Milzer and Byrd(6) reported the successful adaptation of Leon virus to mice using autolyzed brain as an adjuvant in the intracerebral inoculation of mice. This Leon mouse virus is not currently available and further attempts by these workers to repeat this adaptation have thus far been unsuccessful. More recently, however, they have found that "*Salmonella typhimurium* is apparently responsible for the previously reported enhancing effect of autolyzed brain tissue diluent on Lansing virus. Experiments using *S. typhimurium* vaccine for adaptation of strains of poliomyelitis virus to mice are now in progress."†

A technic was recently developed in this laboratory whereby Lansing virus was inoculated directly into the spinal cord of mice(7). It was shown that mice were more susceptible to Lansing infection by this route than by the usual intracerebral method of inoculation. It seemed logical to once more attempt to adapt non-Lansing strains to mice by intraspinal inoculation. This report presents evidence that the Leon strain of poliomyelitis has been thus adapted to mice.

Materials and methods. Leon virus. The Leon virus was obtained from the American Type Culture Collection and had originated in the laboratory of Dr. H. A. Howe of Johns Hopkins Poliomyelitis Center. It was received in its 23rd monkey passage and was given one passage intracerebrally, at a 10^{-3} dilution in a *M. mulatta* monkey in our laboratory before being used in mice. *Immune monkey sera.* Samples of proven Leon, Lansing, and Brunhilde immune monkey sera were obtained through the courtesy of Dr. H. A. Howe from the Johns Hopkins Poliomyelitis Center, Dr. J. Melnick at Yale University, and Dr. W. McD. Hammond at the University of Pittsburgh. In our own laboratory, monkeys were hyperimmunized with 2 cc doses of 20% whole emulsion of monkey cord virus given intramuscularly twice weekly for approximately 14 doses and bled 10 days after the last dose. Some of these sera were not checked for antibody content in monkeys before being used in mouse tests. *Intraspinal inoculation.* The technic of intraspinal inoculation has been previously described.

Virus neutralization test. All sera were inactivated at 56°C for 30 minutes before being serially diluted and mixed with equal parts of the supernatant fluid from a 20% emulsion of Leon mouse passage spinal cords. The mixtures were placed at 4°C for 4 hours before being inoculated intraspinally into mice. Normal monkey serum was included as a control and virus titers were carried out after the 4 hours incubation in the presence of 1/4 dilution of normal serum. In some tests incubation for 30 minutes at room temperature preceded the ice box incubation.

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Animals. Swiss and C_3H mice were used at the age of 3-4 weeks. *Macacus mulatta* monkeys weighing 5-7 lb were used for infectivity tests. Cotton rats were usually young adults and weighed approximately 70 g at the time of inoculation.

Results Primary isolation—8 C_3H mice were inoculated intraspinally with 0.02 ml of a 20% whole emulsion of spinal cord from Leon monkey I-21. There was one death without preceding paralysis on the 7th day. On the 15th day, 2 mice were paralyzed in the hind legs. On the 16th day one mouse was found dead. On the 18th day one mouse was paralyzed in the right front leg, one in the hind leg on the 19th day, 2 others were found dead on the 20th day and the last mouse was found dead on the 21st day. All mice observed with paralysis were sacrificed and their cords harvested for passage.

Intracerebral inoculation of C_3H mice with the same original monkey cord suspension did not cause any paralysis although there were some deaths between the 5th and 11th days. On the 14th day 3 mice developed non-specific symptoms of weakness and ruffled fur and their brains were harvested. Subsequent intracerebral passage of these brains in C_3H mice gave negative results.

Mouse passages. The cords of all mice showing definite paralysis in the first passage were pooled and the supernatant fluid of a 10% emulsion passed in C_3H mice intraspinally. To date this virus has been through a total of 35 such intraspinal mouse passages. The first 5 passages were made in C_3H mice but all subsequent work has been done in Swiss mice which appear to be equally susceptible to the passage virus. Table I shows the morbidity rates and average incubation periods for the early passages.

TABLE I. Incubation Period and Morbidity of Mouse Passages of Leon Virus. Passage of 10^{-1} dilution of mouse cords.

Mouse passage	Incubation period	Morbidity
1	15	100
2	4	70
3	4	100
4	4	100
5	5	90
6	5	85
7	4	100
8	5	100

Identification of passage virus as Leon poliomyelitis. Besides serological and cross immunity experiments to establish the identity of a strain of poliomyelitis other criteria are widely accepted as proof that a particular poliomyelitis virus is probably of human origin. These criteria include its infectivity for monkeys, the typical histopathology produced in the monkey spinal cord and finally its ability to be neutralized by pooled adult human serum. The mouse passage Leon virus has been checked by each of these methods.

(a) *Infectivity for monkeys.* Four monkeys received 1 ml intracerebrally and 1 ml intranasally under ether anesthesia of a 20% whole emulsion of mouse passage No. 8 cords. Fig. 1 shows the subsequent illness in these monkeys. Two out of the 4 developed fever, tremors and finally paralysis of at least 2 extremities after 6 and 7 days' incubation period. These 2 animals when prostrated were sacrificed for virus and histological examination. The other 2 monkeys developed fever and tremors between the 7th and 9th days after inoculation and one (K-19) had weakness of the right arm which did not progress. Cord from monkey K-18 was inoculated intracerebrally at a 10% emulsion into 2 monkeys. After 5 and 9 days these animals came down with fever, tremors and prostrating paralysis. Cords from K-18 and K-21 representing the first monkey passage of mouse virus were examined by Dr. James Peers of the Laboratory of Pathology and found to have typical histopathological lesions of polio of moderate severity at thoracic, lumbar and sacral levels. Subsequent tests for infectivity after intracerebral inoculation of monkeys have been carried out with pooled mouse passages 20, 21, and 22 in an attempt to quantitate and compare the titer intracerebrally in monkeys to intraspinally in mice. Table II shows that if any difference existed in the susceptibility of mice and monkeys to mouse passage Leon virus perhaps monkeys

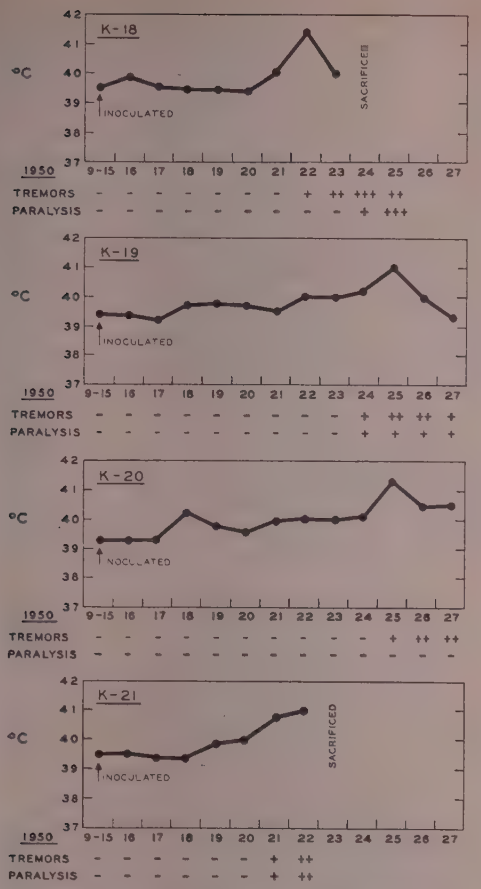

FIG. 1. Results of intracerebral inoculation of monkeys with 8th mouse passage Leon.

were slightly more susceptible. Cord of monkey K-18 (1st monkey passage of mouse virus) was inoculated intraspinally into C_3H mice and the virus thus reisolated in mice and carried through 9 subsequent mouse passages.

(b) *Cross immunity tests in monkeys.* Cord emulsion from first monkey passage of mouse Leon virus was inoculated into 2 fresh monkeys (K-18-2A and K-18-2B) and monkey I-50. Monkey I-50 had previously been hyperimmunized with monkey passage Leon virus and shown to be immune to an intracerebral challenge with the homologous monkey virus. Fig. 2 shows that both fresh monkeys came down with severe paralytic disease while the Leon immune monkey remained normal. A cross-check of the immunity produced by mouse passage Leon virus in monkeys when challenged by monkey passage Leon virus was next carried out. Monkeys K-19 and K-20 which had survived inoculation with mouse passage No. 8 virus with minimum symptoms (see above) were challenged with Leon monkey passage virus together with 2 fresh monkeys (K-39 and K-40). A 10^{-3} dilution of monkey passage No. 24 (monkey I-22) cord was inoculated intracerebrally. Fig. 3 shows that the 2 fresh monkeys developed typical paralytic disease after 6- and 9-day incubation periods, whereas the 2 monkeys convalescent from infection with mouse passage Leon virus failed to develop any symptoms whatsoever. These 2 monkeys had been bled prior to this challenge and both had demonstrable neutralizing antibodies against the mouse passage Leon virus in their serum.

(c) *Virus neutralization tests with mouse passage Leon virus.* The mouse passage Leon virus was tested against serum from a monkey that had been hyperimmunized with monkey passage Leon cord suspensions and proven immune to intracerebral challenge with this same virus. Results are given in Table III. This type of neutralization test was repeated several times with later passages of mouse virus against Leon immune monkey serum obtained from other laboratories with approximately the same quantitative results. Tests against anti-Theiler's virus serum and anti-EMC serum (kindly supplied by Dr. Joel Warren of the Army Medical School) run at the same time were completely negative. Likewise tests against Lansing and Brunhilde immune monkey serums have been negative except in several tests there appeared to be some slight neutralization of mouse passage Leon virus by Lansing immune serum but quantitatively not as great as with Leon serum. Several tests with commercial samples

TABLE II. Titration of Mouse Passages 20, 21 and 22 Leon Virus IS in Mice and IC in Monkeys.

		Dilution of inoculum		
		10^{-1}	10^{-2}	10^{-3}
Mice	IS	4/8*	2/17	0/8
Monkeys	IC	2/2	1/2	1/2

* No. of animals paralyzed over No. inoculated.

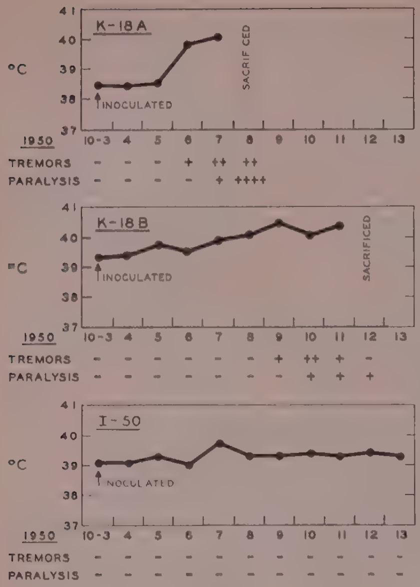

FIG. 2. Intracerebral challenge of 2 fresh monkeys (K 18 A and K 18 B) and one Leon immune monkey (I 50) with 1st monkey passage of Leon mouse adapted virus (monkey K 18 cord).

of gamma globulin (human) which had been shown to contain good neutralization indices against Lansing virus were tested for neutralization of mouse Leon virus but in most instances gave equivocal results. The best index that was obtained was between a 1/4 and a 1/16 dilution of the gamma globulin against 1 to 5 minimum paralyzing doses of mouse Leon virus. A typical protocol for these various neutralization tests is shown in Table IV.

It was felt that these neutralization tests carried out by intra-spinal inoculation of mice were not as clear cut as desired so a neutralization test intracerebrally in monkeys was done using Leon immune monkey serum (obtained from W. McD. Hammond) and normal monkey serum mixed with mouse passage Leon virus (M.P. No. 27) and with monkey passage Leon virus. Undiluted inactivated serum was mixed with supernatant of 20% mouse cord emulsion and 10% monkey cord emulsion. For the monkey virus this represented approximately 100 MPD for monkeys. After 30 minutes at room temperature and 3 hours at 4°C, 1 cc amounts were inoculated intracerebrally into 4 monkeys and in the case of the mouse virus also into groups of mice. Table V shows the results in monkeys and the protocol in Table IV includes the results in mice. The monkey test gave good neutralization of the mouse virus with serum prepared in another laboratory by hyperimmunization with monkey Leon virus.

(d) *Chick embryo inoculation.* Mouse passage No. 10 at a 10% emulsion was inoculated into the yolk sac of 6-day and allantoic sac of 8-day chick embryos. Every 7 days the yolk sac series was passed and transferred while the allantoic sac group were passaged at 6-day intervals. At each passage whole embryos, portions of chorio-allantoic and amniotic membranes and yolk sacs were emulsified in allantoic fluid for the next inocu-

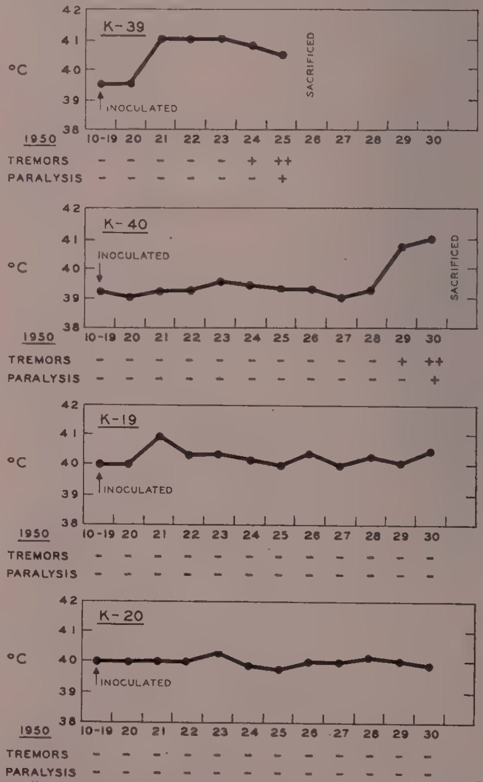

FIG. 3. Monkeys convalescent from intracerebral inoculation with mouse passage virus (K 19 and K 20) and two fresh monkeys (K 39 and K 40) challenged IC with 10^{-3} dilution of monkey passage Leon virus (I 22 cord).

TABLE III. Neutralization Test with Mouse Passage Leon Virus Against Serum of Monkey Immunized with Monkey Passage Leon Virus (IS in Mice).

	Serum dilutions*		
	Undil.	1/4	1/16
Normal monkey	6/9†	5/9	3/8
Leon immune monkey	0/7	1/7	3/10

* Dilutions of serum mixed with 1/5 dilution of mouse passage 9 cords and held at 37°C for 1 hr.

† No. of mice paralyzed over No. inoculated.

TABLE IV. Neutralization Test of Mouse Passage Leon Virus Against Monkey Immune Serum and Human Gamma Globulin (IS in Mice).

	Serum dilutions*			
	Undil.	1/4	1/16	1/64
Normal monkey	7/8†	9/10	7/8	—
Brunhilde immune monkey	7/8	6/6	7/9	—
Lansing ” ”	7/10	3/5	7/9	—
Leon ” ”	3/10	3/7	7/9	—
Gamma globulin	—	2/8	5/9	6/8
Virus titer 1/50‡	—	5/9	—	—
1/250	—	4/10	—	—

* Dilutions of serum mixed with 1/5 dilution of M.P. 27 Leon virus and incubated 30 min at room temperature then 3 hr at 4°C before IS inoculation of mice.

† No. of paralyzed over No. of inoculated mice.

‡ After incubation in 1/4 normal serum serial dilutions made in same diluent for virus titration.

TABLE V. Neutralization Test with Leon Immune Monkey Serum Against Mouse and Monkey Passage Leon Viruses IC in Monkeys.

	Monkey serum*	
Virus	Normal	Leon immune
Mouse passage virus†	3/4	0/4
Monkey ” ” ‡	4/4	1/4

* Undiluted inactivated serum mixed with virus and incubated 30 min at room temperature then 3 hr at 4°C before IC inoculation of 1 cc of mixture in monkeys.

† 1/5 dilution mouse passage #27.

‡ 1/10 ” ” monkey K-39 cord.

lation. In 3 such blind passages there were no specific deaths and no pathological lesions were produced in the chick embryos or membranes. The third passage materials were injected intraspinally into groups of mice with negative results.

Properties of Leon mouse passage virus. (a) *Incubation period, mortality, type of paralysis.* The incubation period in the first mouse passage was 15 days but from the second passage on has been between 4 and 6 days at a 10^{-1} inoculum. After the twentieth passage an occasional mouse would develop paralysis on the third day. After paralysis develops the subsequent mortality varies quite a bit from one passage to another but it has not been unusual for 20% of the paralyzed mice to survive. The total morbidity has varied also from 50% to 100%. In more recent passages the morbidity and mortality have tended to be higher and more consistent. In neutralization tests there is a tendency for a greater proportion of the mice inoculated with serum-virus mixtures to survive after developing paralysis than in routine passage mice. The relative proportion of front leg to hind leg paralysis after intraspinal inoculation with this Leon virus is greater than in mice inoculated by this route with Lansing virus.

(b) *Titration of mouse virus.* Virus titers of mouse cords by the intraspinal inoculation in mice have been consistently low, usually a 50% mortality not being obtained beyond a 1/50 dilution of cords. All attempts to infect mice intracerebrally have thus far been negative, yet this same virus emulsion promptly and readily caused typical poliomyelitis after intracerebral inoculation of monkeys. Likewise attempts to infect cotton rats by intracerebral inoculation have been negative. Intraspinal inoculation of cotton rats has not given consistent results although paralytic disease has been produced several times. In each instance inoculation of paralyzed cotton rat cord emulsion intraspinally into mice was negative. Attempts at cotton rat passage have been equivocal. One attempt at intraspinal infection of cotton rats with monkey Leon virus gave mild paralytic symptoms which could not be maintained on passage.

Repeat of primary adaptation to mice. Cord of monkey 122 representing the same monkey passage of Leon virus as used in the original adaptation experiment but from a different monkey was used to inoculate mice intraspinally. The first group of C_3H mice had 2 paralyzed out of 10 on the 30th day. Second passage in C_3H mice had 5 paralyzed out of 12 with the earliest symptoms on the 9th day. Starting with the 3rd passage, Swiss mice were used and the incubation shortened to 5 days. This substrain has been passed through

16 passages intraspinally in mice and has continued to have an incubation period of 5 to 6 days. Mouse passage 15 cords were inoculated intracerebrally into 2 monkeys (K80 and K81) in a 20% emulsion. Both monkeys developed fever, marked tremors and hind leg paralysis starting on the 7th day.

Discussion and summary. Leon type of human poliomyelitis virus has been adapted to mice from original monkey cord virus by use of an intraspinal technic of inoculation. This mouse adapted Leon virus has been serially passed through mice for 35 passages and causes flaccid paralysis, more frequently in the front legs, after an incubation period of 4 to 6 days. A second similar adaptation has been accomplished and this strain carried through 16 mouse passages. The mouse virus causes paralysis only after intraspinal inoculation of mice, being negative when given intracerebrally and is of low virulence thus far. Apparently this virus is still as virulent for monkeys as it is for mice. Proof that this mouse virus is indeed Leon poliomyelitis virus rests on its ability to cause typical clinical and histopathological disease after intracerebral inoculation of monkeys, cross immunization against monkey Leon virus in monkeys and cross neutralization tests in mice and in monkeys. The virus also fails to propagate in the chick embryo. It is neutralized by human gamma globulin in low dilutions. Thus far attempts to adapt the Brunhilde type of human poliomyelitis virus to mice by this same method have been completely negative.

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Megaloblastic Anemia in Pregnancy. Remission Following Combined Therapy with Ascorbic Acid and Vitamin B_{12}.* (19033)

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A recent report by Thompson and Ungley (1) has reviewed many of the clinical and hematologic features of megaloblastic anemia in pregnancy. These authors emphasize the lack of response to vit. B_{12}. Similar observations had previously been reported by Day, Hall and Pease(2). In this respect the megaloblastic anemia in pregnancy is similar to megaloblastic anemia experimentally produced in monkeys(3) and to the megaloblastic anemia in some infants(4). Remissions are regularly produced in these three as well as other megaloblastic anemias by folic acid therapy. The megaloblastic anemia in some infants and as experimentally produced in monkeys is related to a deficiency in ascorbic acid and can be prevented by a normal intake of this substance. May(3) has suggested that the ascorbic acid deficiency may interfere with the conversion of folic acid to the citrovorum factor as Nichol and Welch(5) have shown that ascorbic acid augments the conversion of folic acid to the citrovorum factor *in vitro*. It is entirely possible that megaloblastic anemia in some infants and that produced in monkeys results from a deficiency of the citrovorum factor which in turn is produced by altered folic acid metabolism resulting from ascorbic acid deficiency.

Observations on 5 patients with megaloblastic anemia in pregnancy indicate that a similar mechanism may hold for the pathogenesis of this anemia. Megaloblastic anemia in pregnancy is refractory to vit. B_{12}. Two of the cases to be reported confirm this finding. The pregnancy megaloblastic anemia may be related to ascorbic acid deficiency. A seasonal incidence was present in that all 5 cases appeared in April and May. Lund (6) has shown decreased plasma ascorbic acid values in normal pregnancy during winter and spring months when exogenous sources of ascorbic acid are apt to be low. In 3 of the patients on whom determinations for plasma ascorbic acid were run, decreased or zero values were obtained. To test the analogy further, 3 of the patients were treated with vit. B_{12} and ascorbic acid separately and then in combination. Combined therapy in each of the 3 patients produced a complete hematologic remission where separate therapy with ascorbic acid or vit. B_{12} was ineffective. A fourth patient was first seen after 10 days therapy with vitamin B_{12} during which time the anemia had become progressively more marked. This patient responded to combined folic acid and transfusion therapy. No therapy was given the fifth patient. A spontaneous remission followed delivery. The diagnosis of megaloblastic anemia was made in each case by bone marrow biopsy.

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* This study was carried out under a grant from Eli Lilly and Co.

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Case 1. D.H. Age 23 Para 2-0-0-2. The diagnosis of megaloblastic anemia was made 7 days before delivery. The hemoglobin was 8.8 g %, erythrocytes 2.66 million, hematocrit 23% and reticulocytes 1.2%. Free acid was present on gastric analysis. The plasma ascorbic acid level was 0.3 mg %. Daily administration of 500 mg of ascorbic acid by intramuscular injection produced a maximum reticulocyte response of 3.6% on the fifth day. The marrow was unchanged at the completion of this test period. While on ascorbic acid therapy the hemoglobin dropped to 6.2 g %, erythrocytes to 1.76 million and the hematocrit to 18%. A daily dose of 30 μg of vit. B_{12} was added to the ascorbic acid therapy and was followed by a maximum reticulocyte response of 25.4% 9 days after the onset of combined therapy. The marrow reverted to normoblastic development. Rapid clinical and hematologic improvement followed. On discharge from the hospital 27 days post partum the hemoglobin was 11.6 g %, erythrocytes 2.93 million and hematocrit 33%. Later checks showed a return to normal hematologic values.

Case 2. D.H. Age 24 Para 3-0-0-3 (Same patient as case 3). The patient was followed throughout this pregnancy. A diagnosis of megaloblastic anemia was made in the ninth month of pregnancy. Hemoglobin was 8.1 g %, erythrocytes 2.54 million, hematocrit 23% and reticulocytes 0.6%. After the initial workup, daily administration of 500 mg of ascorbic acid was started. A trial period of 4 days was allowed at the end of which time the marrow was still megaloblastic. No reticulocyte increase was observed. The hemoglobin had dropped to 6.4 g %, erythrocytes to 1.86 million and hematocrit to 20%. Thirty micrograms of intramuscular vitamin B_{12} was combined with the ascorbic acid and 9 days later a maximum reticulocyte response of 15.4% was noted. Marrow reversion to normoblastic pattern was noted after the institution of combined therapy. Clinical and hematologic response again was rapid. Twenty-eight days after delivery the hemoglobin was 11 g %, erythrocytes 3.72 million and hematocrit 37%.

Case 3. M.S. Age 18 Para 0-0-0-0. Megaloblastic anemia was diagnosed in the eighth month of pregnancy. Plasma ascorbic acid level was zero. Hemoglobin was 8.6 g %, erythrocytes 2.14 million, hematocrit 25% and reticulocytes 0.4%. Treatment with 30 μg of intramuscular vit. B_{12} over a 6-day period did not produce a reticulocyte response. At the end of this 6-day period the bone marrow was unchanged. The hemoglobin dropped to 7.6 g %. The combination of 300 mg of ascorbic acid with vit. B_{12} was followed in 4 days by an increase in reticulocytes to 13.4%. Seventy-two hours after the onset of combined therapy the bone marrow was normoblastic. Twenty days after admission and while still undelivered the hemoglobin was 10.4 g %, erythrocytes 2.67 million

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TABLE I. Summary of Data on 5 Cases of Megaloblastic Anemia in Pregnancy.

	Date	Comment	Hemoglobin, g %	Erythrocytes, million μ^3	Hemato-crit, %	Reticulo-cytes, %
D.H.	2-28	Initial workup	8.8	2.66	23	1.2
	3-7	Day of delivery	7.4	1.75		1.3
	-8	Ascorbic acid therapy begun				
	-13		6.2	1.76	18	3.5
	-14	B_{12} therapy started				
	-23	Max retic response	7.5	2.11	24	25.4
	4-3	Discharge	11.6	2.93	33	3.4
D.H.	1-12	6th month of pregnancy	12.5	3.35	36	1.2
	4-12	Initial workup	8.1	2.54	23	.6
	-17	Ascorbic acid therapy begun				
	-18	Day of delivery				
	-20		7.1	1.86	20	1
	-21	B_{12} therapy started				
	-30	Max retic response	8.6	2.23	29	15.4
	5-16	Discharge	11.1	3.72	37	2.8
M.S.	5-9	8th month of pregnancy	8.6	2.14	25	.4
	-15	Vit. B_{12} therapy started	7.6			
	-21	Ascorbic acid therapy started				
	-25	Max retic response	10.7			13.4
	6-18	Day of delivery	13.5	3.05	37	1.8
I.M.	5-4	Admitted to hospital	5.1	1.41		
	-6	Crude liver and B_{12} started				
	-12	Transfusion 500 cc				
	-13	Day of delivery, folic acid	6.9	1.53	15	.3
	-21	Transfusion 500 cc	11	3.16		
S.K.	5-12	Day of delivery				
	-13	Initial workup	8.4	2.83	27.5	.2
	-23	Max retic response				5.5
	6-24		11.5	3.38	39	.6

and hematocrit 30%. At delivery 6 weeks after admission the hemoglobin was 13.5 g % and hematocrit 37%.

Case 4. I.M. Age 34 Para 1-0-2-1. Megaloblastic anemia was diagnosed on the day of delivery though the patient had been hospitalized and treated with vit. B_{12} and crude liver extract for 10 days prior to diagnosis. Following delivery an adequate response was obtained with folic acid and blood transfusions. The initial hemoglobin was 6.9 g %, erythrocytes 1.53 million, hematocrit 15% and reticulocytes 0.3%. Aside from the demonstration that vit. B_{12} administration had not prevented the appearance of this megaloblastic anemia, little information was obtained.

Case 5. S.K. Age 42 Para 7-2-2-9. The diagnosis of megaloblastic anemia was made on the day following delivery. The hemoglobin was 8.4 g %, erythrocytes 2.83 million, hematocrit 27.5% and reticulocytes 0.2%. There was free acid on gastric analysis. Plasma ascorbic acid value was zero. Inadvertently no therapy was given this patient but a spontaneous remission followed delivery. A maximum reticulocyte response of 5.4% appeared 11 days after delivery. When seen 43 days after delivery the hemoglobin was 11.5 g %.

The essential data are summarized in Table I. Other tests were made on these patients which are of some interest. In 4 patients on whom stool specimens were obtained before therapy there was no increase in urobilinogen excretion. Serum iron determinations made prior to therapy on 4 patients showed values of 318, 348, 365 and 268 γ %. The iron binding capacity of the serum was less than 50 γ % in the same 4 patients. Following combined therapy there was a sudden decrease in serum iron values to less than 50 γ % and increases in the iron binding capacity. Normal erythrocyte protoporphyrin values were obtained on all 5 patients prior to therapy. After therapy these values increased as the bone marrow reverted to normoblastic activity.

It is possible that ascorbic acid therapy alone over a long enough period of time would produce a remission. In Case 1 a suggestive increase in reticulocytes occurred but therapy was not continued long enough to warrant any conclusions. It is probable that megaloblastic anemia in pregnancy can be prevented by adequate ascorbic acid intake during pregnancy.

Summary. Five cases of megaloblastic anemia in pregnancy are reported. The relation of this anemia to a deficiency in ascorbic acid is suggested by the effectiveness of combined ascorbic acid and vit. B_{12} therapy where vit. B_{12} alone was ineffective. Two patients were refractory to adequate trial with vit. B_{12}. One of these patients later had a complete remission when ascorbic acid was added to the therapy. The other responded adequately to folic acid therapy and blood transfusions. Two patients were treated for short periods of time with ascorbic acid alone. A minimal response in reticulocytes was observed in one. Subsequent addition of vit. B_{12} to the therapy after saturation with ascorbic acid produced a complete hematologic remission. One patient had a spontaneous remission after delivery. In 3 patients combined therapy with ascorbic acid and vit. B_{12} produced a complete remission.

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Fiber Formation in Trypsinogen Solutions: An Electron Optical Study.*

(19034)

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Recently the author(1) reported a study of the structure of elastic tissue with the electron microscope which emphasized a seemingly specific effect of trypsin on elastic fibers in releasing large numbers of characteristically coiled threads. These were composed of pairs of smooth filaments each about 70 A in diameter coiled in a tight, regular helix with a pitch of 500 A and a width of 120 A. Numerous unwound filaments were also observed. It was also reported that electron micrographs of freshly prepared trypsin solutions dried on the supporting film did not show the coiled threads. More recently Franchi and DeRobertis(2) reported the appearance of coiled threads, resembling those described above, in solutions of Armour's crystalline trypsin after incubation—in the absence of elastin—whereas, after sterilization of the trypsin solution by Seitz filtration, no such elements were found. They also reported fresh trypsin solutions to be devoid of fibers. Because a few bacteria and flagella were found in the non-sterilized preparations, they suggested that the coiled threads observed by Gross and themselves were degradation products of tryptically digested flagellae. In a second paper(3) these authors described the appearance of coiled threads in a culture of *Bacillus brevis* incubated in Armour's crystalline trypsin. This organism was found as a contaminant of the enzyme.

This paper describes a reinvestigation of the

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* From the Research Laboratories of the Medical Clinic, Massachusetts General Hospital, the Department of Medicine, Harvard Medical School, and the Massachusetts Department of Public Health, Boston, Massachusetts. This is publication No. 123 of the Robert W. Lovett Memorial for the study of crippling disease, Harvard Medical School. The expenses of this investigation were defrayed in part by a grant from the United States Public Health Service.

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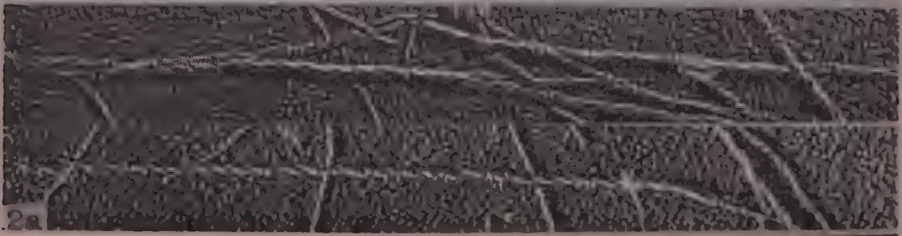

2a

FIG. 1. Electron micrograph of coiled threads and filaments present in fresh crystalline trypsin solutions. 24,600 ×.

FIG. 1a. Enlargement of small area of Fig. 1. 129,500 ×.

FIG. 2. Electron micrograph of coiled threads and filaments produced in trypsinogen solution. 41,000 ×.

FIG. 2a. Enlargement of two regions of Fig. 2. 70,000 ×.

origin of the coiled threads previously observed after tryptic digestion of elastin.

Experimental procedures and results. Careful reexamination of specimens prepared by depositing a drop of fresh 0.1% water solutions of Armour's crystalline trypsin on the collodion supporting film of the specimen grid, draining after 5 minutes and shadowing with chromium, revealed the vague outlines of what might be fibrous material covered with a thick amorphous layer. Centrifugation of such trypsin solutions for 20 to 30 minutes at 4000 RPM in the International centrifuge produced a small amount of white sediment. This was drained of supernatant, redispersed in water and prepared for electron microscopy as described above. Examination showed large numbers of helically-coiled threads identical with those described previously by the author (Fig. 1). Thus, the controls of the trypsin solutions, as performed both by Gross and by Franchi and DeRobertis, were obviously inadequate; the coiled threads and filaments were pre-existent in the unincubated "crystalline" trypsin and required concentration and freeing from the amorphous material before they could be detected. Five separate batches of Armour's crystalline trypsin contained large numbers of coiled threads and filaments. Visible sediments were obtained on centrifugation of both water and bicarbonate-buffered solutions in all instances. These structures were not found in Armour's "Tryptar", a highly purified and Seitz-filtered crystallized trypsin.

Few, if any, bacteria were seen in the electron micrographs, and in fact one of these trypsin preparations was proven sterile by thioglycollate and blood plate cultures (kindly prepared by Dr. L. Dienes) while the other 4 batches produced very few colonies.

Seitz-filtered water solutions of Armour's crystalline trypsin were free of threads and filaments and, in fact, were shown to be devoid of tryptic activity (gelatin digestion) when small quantities (10 cc of 0.1% solution) were filtered, probably because of adsorption of the enzyme. This method of sterilization was used by Franchi and DeRobertis and probably accounts for their failure to find coiled threads in sterilized preparations; they were filtered out.

Fragmented ligamentum nuchae, purified and native, was incubated with the clear supernate of centrifuged Armour's crystalline trypsin and Armour's "Tryptar" in bicarbonate-buffered (pH 7.8) solution at 37°C. The enzyme solutions were also tested for proteolytic activity. Electron microscope examination of the centrifuged sediments of such preparations after shadowing revealed no coiled threads. It was therefore concluded that purified, crystallized trypsin does not liberate these structures from elastic tissue.

Because of the existence of these characteristic fibrous elements in the crystalline trypsin, it seemed desirable to test the precursor substances.

Water solutions of crystalline trypsinogen were centrifuged as described above, and both supernatant and sediment (extremely small) proved to be devoid of the specific fibrous elements.[†] One per cent solutions of trypsinogen (same batch) in 0.4 M borate buffer at pH 8.0 were seeded with a few crystals of "Tryptar" (to activate the trypsinogen)[‡] and incubated at 5°C for 3 to 7 days and their sediment examined after centrifugation at 30,000 RPM for 30 minutes in the Spinco preparative centrifuge.§ Large numbers of typically coiled threads and uncoiled filaments were observed (Fig. 2). Examination of similar sediments prepared within 15 minutes of solution of the trypsinogen in buffer revealed a rare coiled thread or filament. Incubation of trypsinogen at 5°C in 0.4 borate buffer at pH 5.2 failed to produce any fibrous structures. This experiment was then repeated under sterile conditions. The "Tryptar"-seeded trypsinogen solution buffered at pH 8.0 was passed through an ultra-fine, sterilized, sintered glass filter and checked by thioglycollate and blood plate cultures for bacterial contamination immediately following filtration and after five days of incubation at 5°C. The solution proved to be sterile throughout the experiment. No coiled threads or filaments were found in the fresh filtrate. After incubation for 5 days the solutions were centrifuged and the sediments examined. Large numbers of long, typically coiled threads and filaments were observed in all fields.

[†] Since submission of this manuscript, two recent lots of Worthington's crystalline trypsinogen were shown to contain large numbers of coiled threads. These preparations were centrifuged and passed through ultra-fine sintered glass filters and reexamined prior to incubation. The filtrates were clear of threads. An Armour preparation of crude crystallized trypsinogen contained no demonstrable threads and gave large masses of them after incubation.

[‡] Subsequent work indicates that trypsin seeding is not necessary for this process and, in fact, coiled threads will form in the presence of large quantities of crystallized pancreatic and soy bean trypsin inhibitors.

Thus, it would appear that the characteristically coiled threads, described as components of elastic tissue by this author and as components of bacterial flagellae by Franchi and DeRobertis, are in fact fibrous structures arising from solutions of purified, crystallized trypsinogen during incubation under certain conditions. There exists the interesting possibility that this phenomenon may represent a fibrous transformation of trypsinogen or a component or contaminant thereof. Such transformations are known to occur in solutions of insulin(4), actin(5), and tropomysin(6). Further studies on the characterization of these fibrous units and the conditions for their production will be reported in detail elsewhere.

Summary. The coiled threads and filaments described by Gross as essential components of elastin, liberated from the tissue by tryptic digestion, and later described by Franchi and DeRobertis as products of tryptically digested bacterial flagellae have been demonstrated to be present in Armour's crystalline trypsin, but not in the more highly purified product "Tryptar," and can be produced by incubation from clear, sterile solutions of crystalline trypsinogen. This process probably represents a fibrous transformation of trypsinogen, a component or contaminant thereof.

The author wishes to thank Miss Zelda Kaufman for excellent technical assistance and the Research Division of Armour and Company, for providing purified proteolytic enzymes and samples from various stages of trypsin manufacture.

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Received August 27, 1951. P.S.E.B.M., 1951, v78.

[§] Because of the very small (oft-times invisible) sediment, the walls of the centrifuge tube had to be scrubbed thoroughly in 1 cc distilled water. Higher trypsinogen concentrations produced visible sediments.

Evidence for a Sodium Retaining Factor in Toxemia of Pregnancy.* (19035)

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Toxemia of late pregnancy is a clinical problem which still remains to be defined in terms of etiology and pathological physiology. Practical management has taken diverse forms and has evolved from one plan to another, but

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TABLE I. Excretion of a Sodium Retaining Factor in Late Pregnancy.

Patient	Type of pregnancy	Date	17-ketosteroid, mg/24 hr	11-oxysteroid, mg/24 hr	Sodium factor in µg equiv. DCA/24 hr	Remarks
VF	Toxemic	11/16-18/50	7.7	1.193	228	LMP: 4/20/50 EDC: 1/27/51
		12/1-3	4	.591	604	Toxemia began 10/20/50
		12/10-12	5.3	.393	60	Caesarean section 12/8/50
EB	"	11/11-13	8.1	.416	1008	LMP: 2/22/50 EDC: 11/29/50
		11/17-19	7.2	.902	463	Toxemia began 9/15/50; induction of labor 11/16/50; stillborn infant
EJ	Toxemic-diabetic	5/29	11.6		756	LMP: 10/6/50 EDC: 7/13/51
		7/4	12		48	Toxemia began 5/20/51; caesarean section 7/1/51
LM	"	1/14/51	7.2	.900	21	LMP: 6/15/50 EDC: 3/25/51
		2/22	12.6	.631	32	Toxemia began 2/20/51
		3/2	13.5	.977	322	Caesarean section 3/2/51
		3/3	8.8	1.453	72	Postpartum eclampsia
		3/4	7.6	1.428	43	
NR	"	3/28	9.8	.806	278	LMP: 12/6/50 EDC: 9/13/51
		7/1	21		653	Toxemia began 5/20/51
		8/10			106	Caesarean section 8/8/51
EO	Toxemic	5/27	20.6		490	LMP: 8/15/50 EDC: 5/22/51
		5/30			226	Toxemia began 12/20/50
		6/4	10		48	Caesarean section 5/28/51
ML	Toxemic-diabetic	6/22	15.8		202	LMP: 11/15/50 EDC: 8/22/51
		7/29			422	Toxemia began 6/30/51
		7/30	16.4			Caesarean section 7/30/51
		8/4			106	
NN	Normal		5.4		36	In middle of last trimester
NM	"		18.4		61	" " " " "
SS	"				100	Beginning of " "
NS	"				104	In middle " " "

all have as their ultimate goal, the control of edema, reduction of blood pressure, maintenance of adequate nutrition, especially of proteins, and prevention of eclampsia. In this complex of clinical manifestations there exists strong suggestive evidence of the presence of pathological factors exerting abnormal control over electrolyte and fluid balance. It is quite logical, therefore, that the function of the adrenal cortex should be investigated in this connection.

In the present study an attempt has been made to measure the quantitative urinary excretion of various steroid fractions thought to be of adrenal origin, in toxemic as well as normal pregnancies, with special attention to the sodium retaining function of these urinary compounds. 17-Ketosteroids have been measured by a standard chemical method after extraction with chloroform(1). 11-Oxysteroids or "glucocorticoids" have been measured chemically by Mason's modification(2) of the method of Daughaday and Williams (3). Sodium retention has been measured by means of a biological assay procedure developed by Kagawa, Shipley and Meyer(4), using adrenalectomized rats. The rats are used for the test 24 hours after adrenalectomy. The test period of 6 hours is initiated by the subcutaneous injection of desoxycorticosterone acetate or urinary extract, followed 1 hour later by the subcutaneous injection of 8.29

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mg of sodium in the form of sodium chloride. Immediately following the sodium chloride injection, the urethra is ligated and four hours later the animal is sacrificed, the urinary bladder is removed, and its contents and washings analyzed for sodium. The procedure is standardized against pure desoxycorticosterone acetate and results are reported in microgram equivalents of desoxycorticosterone acetate per 24 hours. The method has been found to be sensitive to variations as small as 2 μg of desoxycorticosterone acetate.

Urine collections covering 24- or 48-hour-periods have been made on patients exhibiting the clinical manifestations of toxemia as well as on women with normal pregnancy. Because of the well recognized tendency for diabetic pregnancies to become toxemic, several diabetic pregnant patients have been studied with the advantage that they have been observed before and during their transition into the toxemic state. In one instance it has been possible to predict from the urinary assay figures, the appearance of toxemia before the usual manifestations have appeared. The results are to be seen in Table I.

It is evident that the usual assays of the common steroidal fractions performed by chemical methods have given no consistent indication of abnormality in toxemic as compared to normal pregnancies(5,6). In the few instances in the present study in which formaldehydogenic steroids and the sodium retaining factor have been measured in the same specimens, no significant rise in the former has been noted. The sodium retaining factor, however, has shown a sharp and striking rise in every instance, and has often preceded the appearance of edema and hypertension. The identity of the compound, or compounds, involved in this phenomenon is unknown but it is chloroform soluble, presumably steroidal in nature, and possibly of adrenal origin. A placental source has not been excluded, however, and studies on this point are being conducted at present and will be included in a later publication. The action of the chloroform extracted fraction on sodium balance studies suggests that the active material is a desoxycorticosterone-like substance. No clue is provided in these studies as to the nature of the physiological mechanism which might lead to an adrenal stimulation of this type in some pregnancies and especially in diabetic pregnancies, but it does seem probable that all of the clinical manifestations of the toxemia complex may be explainable on this basis.

Summary. A marked rise in excretion of a sodium retaining factor has been observed in toxemic pregnancies in both diabetic and non-diabetic patients. The increase has been observed in one patient before other toxemic symptoms have become evident. No parallel increases in 11-oxysteroid excretions were found in these patients.

The authors gratefully acknowledge the technical assistance of Miss Dorothy Hagedorn and Miss Phyllis Helmer in preparing the urinary extracts and determining the 17-ketosteroid levels.

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Response of Infant and Adult Mice to Lymphocytic Choriomeningitis Virus Infection. (19036)

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Immature animals are more susceptible than adults to many viral infections(1-10). The Coxsackie viruses are one extreme expression of this general rule(11) to which the Lansing strain of poliomyelitis virus is an exception(12,13). As the present report shows, lymphocytic choriomeningitis appears to be a second exception.

Unsuitability of infant mice for isolation of virus. The original observation was made while recovering strains of lymphocytic choriomeningitis virus (LCM) from a specimen of human cerebrospinal fluid and from a number of house mice. All these samples proved to be infectious for adult mice and the cerebrospinal fluid was infectious as well for guinea pigs. The cerebrospinal fluid was inoculated intracerebrally into two 200-400-g guinea pigs, five 4-week-old mice, and eleven 1- to 3-day-old mice The animals were of the Albany standard strains. The dose was 0.1 ml for the guinea pigs, 0.03 ml for the older mice, and 0.01 ml for the infant mice.

Only 1 of the 11 infant mice died (Table I). It was found on the 10th day and was partially eaten when discovered. All of the adult mice died within 9 days and both guinea pigs within 15 days. Both groups had exhibited characteristic signs of lymphocytic choriomeningitis and microscopic examination revealed histologic changes consistent with that diagnosis. The infectivity of brain suspensions of the guinea pigs and adult mice was neutralized by sera from monkeys hyperimmunized with LCM virus strain 4707.* Similar results were obtained with organ suspensions from the 5 house mice. The brains were ground separately; the liver and spleen of each were pooled before grinding. Ten per cent (by weight) suspensions were made. The diluent was 10% physiologic saline-nutrient broth solution containing 1000 units of penicillin and 1000 μg of streptomycin per milliliter. Dose and route of inoculation were the same as used with the cerebrospinal fluid. Of the 73 infant mice so treated, 7 died during the period when mature mice characteristically manifest signs of choriomeningitis. Six others died after a month or more. In contrast, 44 of the 45 adult mice died within 8 days, 38 following signs of LCM infection.

The agents isolated from each of the tissue suspensions were identified as LCM viruses by neutralization tests with hyperimmune monkey serum. Thus of 50 adult mice injected with samples from the various sources for primary isolation of LCM virus, 43 died or showed typical signs. Of 84 infant mice injected with the same specimens only 14 died, 6 of them approximately a month after

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* Lymphocytic choriomeningitis virus strain 4707 was isolated in Raleigh, N. C., by Mrs. Margaret Douglass Umphlett and was identified in the Grasslands Hospital Laboratory, Valhalla, N. Y., by Dr. Gilbert Dalldorf.

TABLE I. Primary Isolation of Lymphocytic Choriomeningitis Virus in Adult and Infant Mice.

Inoculum	Adult mice* Total No.	Adult mice* Day of death	Adult mice* No. survivors	Infant mice† Total No.	Infant mice† Day of death	Infant mice† No. survivors
Human cerebrospinal fluid	5	9 9 (9)‡(9) (9)	0	11	10	10
House mouse						
No. 1 Brain	5	1 1 1 8 (7)	0	5		5
Liver and spleen	5	(7) (7) (7) (7)	1	11	6 6 35	8
No. 2 Brain	5	(7) 7 7 7 7	0	8	13	7
Liver and spleen	5	(7) (7) (7) (7) 8	0	12	6 6 35	9
No. 3 Brain	5	4 4 (7) 7 7	0	7	8	6
Liver and spleen	5	(7) (7) (7) (7) 7	0	6	8	5
No. 4 Brain	5	7 7 7 7 7	0	7		7
Liver and spleen	5	(7) (7) (7) (7) 8	0	9	32 35 35 38	5
No. 5 Liver and spleen	5	(7) (7) (7) (7) 7	0	8		8
Total	50		1 = 2%	84		70 = 83%

* 4 wk old. Dose, .03 ml, inoc. intracer.
† 1 to 3 days old. Dose, .01 ml, inoc. intracer.
‡ Number in parentheses signifies animal killed after typical convulsions.

TABLE II. Results of Challenge* with Established Strain of Lymphocytic Choriomeningitis Virus (49189) in Infant Mice† Surviving Inoculation of Infected Tissues.

Original inoculum	Reinoculation—No. days after primary inoc.	Reinoculated mice Total No.	Reinoculated mice Day of death	Reinoculated mice No. survivors	Control mice‡ Total No.	Control mice‡ Day of death	Control mice‡ No. survivors
House mouse							
No. 1 Brain	42	5		5	5	1 1 6 6 6	0
Liver and spleen	42	8	6	7			
No. 2 Brain	42	7	1	6			
Liver and spleen	42	9		9			
No. 3 Brain	47	6		6	10	1 6 6 6 6	0
Liver and spleen	47	5		5		6 7 7 7 9	
No. 4 Brain	43	7	(6)§	6			
Liver and spleen	43	5	1	4			
No. 5 Liver and spleen	43	8	1	7			
Total		60		55 = 91.6%	15		0

* Challenge dose is .3 ml by intracer. route of .1 % suspension of virus strain 49189.
† 1 to 3 days old at time of first inoculation.
‡ Same age as test mice.
§ No. in parentheses signifies animal killed after typical convulsions.

inoculation.

Challenge of infant survivors. The surviving infant mice were tested for active immunity from 42 to 47 days after the original inoculation. They were then inoculated intracerebrally with 0.03 ml of an 0.1% suspension of an established strain of lymphocytic choriomeningitis virus (49189).† Normal mice of approximately the same age were similarly inoculated and served as controls. All but 2 of the previously inoculated mice were resistant, while all the control mice died (Table II).

Relative resistance of infant mice to second passage strains. Second passages of 4 of the strains isolated from house mice were made in parallel in infant and adult mice. The inoculum was brain, and in one instance, pooled liver and spleen, all from first passage adult mice. The conditions of the experiment were otherwise identical with those of the first. The results duplicated those obtained

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† This is the original strain of lymphocytic choriomeningitis virus (designated E371) which was received in 1949 from Dr. Charles Armstrong, National Institutes of Health, Bethesda, Md. It had been through 371 mouse passages in his laboratory and 9 mouse passages in this laboratory.

TABLE III. Response in Adult and Infant Mice to an Established Strain of Lymphocytic Choriomeningitis virus (49189).

	Adult mice*					Infant mice†				
Date of test	Total No.	Virus dilution	Day of death	No. survivors	Mean survival time (days)‡	Total No.	Virus dilution	Day of death	No. survivors	Mean survival time (days)‡
11/14/50	5	10^{-3}	(6)§(6) (6) (6) (7)	0	6.2	5	10^{-3}	8 10 13 13 13	0	11.4
11/30	5	10^{-3}	(6) 7 7 7 7	0	6.8					
5/11/51-A	9	10^{-2}	(6) (6) (6) 6 6 7 7 7 7	0	6.4	8	10^{-2}	10 10 10 10 10 10 10	1	10
	10	10^{-4}	5 7 (7) (7) (7) (7) (7) 10 10 10	0	7.7	8	10^{-4}	10 10 10 12 12	3	10.8
5/11/51-B						8	10^{-2}	10 10 10 10 10	3	10
						8	10^{-4}	10 11 11 11 11 11	2	10.8
Total	29			0	6.7	37			9 = 24.3%	10.6

* 4 weeks old. Dose .03 ml, inoc. intracer.
† 1 to 3 days old. Dose .01 ml, inoc. intracer.
‡ Mean survival time computed only for mice that died.
§ No. in parentheses signifies animal killed after typical convulsions.

previously. Of a total of 41 infant mice inoculated, 3 died 2 weeks later, 2 more died at the end of a month, and the rest survived. When challenged by the intracerebral route with mouse-adapted strain 49189, all but 3 proved immune. Of the 35 adult mice, however, 31 died within 8 days, and one more on the 13th day. None of the 3 adult survivors was immune to LCM strain 49189.

Relative susceptibility of infant mice to a mouse-adapted strain. Adult and infant mice were inoculated into the brain with 0.03 and 0.01 ml, respectively, of dilutions varying from 1% to 0.01% of the mouse-adapted LCM strain 49189. None of the mice in the adult group survived (Table III). The mean survival time was 6.7 days. Nine (24.3%) of the infant mice survived. The mean survival time was 10.6 days.

Discussion. Traub(14,15) has shown that in mouse colonies infected with lymphocytic choriomeningitis virus the adult carrier rate is high but the death rate low. The disease is transmitted *in utero*. The young commonly survive, become immune without manifesting any signs of illness, but excrete virus for long periods of time. In this manner the infection persists without destroying the colony.

In this study it was found that mouse adaptation apparently may enhance the virulence of the agent beyond the ability of previously exposed infant mice to withstand it. The human disease, however, is thought usually to be due to contact with infected house mice(16). The virus is presumably only one passage removed from the wild state when recovered from patients. This might explain the observation that the strain isolated from human cerebrospinal fluid behaved like those obtained from the house mice. It is consonant with the findings that infant mice were relatively resistant to second as well as primary passage strains derived from the house mice. We know that the relative resistance of infant mice to LCM virus in these experiments was not due to humoral immunity passively acquired *in utero* from an immune mother since they came from normal stock.

The relative lack of susceptibility of infant mice to Lansing poliomyelitis virus(12,13) is interesting in view of the gradually increasing frequency and severity of infantile paralysis in older persons. In this respect the present

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trend in the epidemiology of poliomyelitis is comparable to the epizoology of lymphocytic choriomeningitis in mice. Mouse encephalomyelitis is also being explored along these lines.

Summary and conclusions. Freshly isolated strains of lymphocytic choriomeningitis virus induced inapparent infection and immunity in infant mice. A mouse-adapted strain, however, caused obvious signs of infection and a high mortality rate among infant mice. Adult mice were fully susceptible to both freshly isolated and mouse-adapted virus. These findings are in keeping with the epizoology of the disease in naturally infected mouse colonies.

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Changes in Adrenal and Pituitary Concentrations of I^{131} and P^{32} Following Thyroidectomy.* (19037)

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It has been reported that an increased concentration of radioiodine I^{131} occurs in the pituitary and the adrenals of rats subjected to prolonged propylthiouracil treatment(1). This study was undertaken to determine whether a similar response occurs in total thyroidectomy, and by the uptake of radiophosphorus P^{32} to determine if the rate of phosphate utilization is altered in those organs.

Methods and procedure. Twenty young male albino rats weighing between 60 and 100 g were randomly divided into an experimental (Group I) and a control group (Group II). The experimental group was thyroidectomized and maintained with the control animals on regular laboratory Rockland rat ration for a period of 70 days following thyroidectomy. Twenty fully mature male albino rats weighing between 250 and 300 g were similarly divided into an experimental (Group III) and a control group (Group IV). The experimental group was thyroidectomized and maintained with the control animals under identical food, water and housing conditions as the younger rats of Groups I and II, for the same period of time. Three hours before sacrifice, a randomly selected one-half of each group (Groups I-IV) was injected intraperitoneally with 200 microcuries of radioactive iodine I^{131} in 2.5 ml of normal saline. The other half of each group of animals was injected intraperitoneally with 100 microcuries of radioactive phosphorus P^{32} in 2.5 ml of normal saline. At sacrifice the adrenals, pituitary, thymus, a portion of muscle and the thyroids of the control groups (Groups II and IV) were removed, weighed and the entire adrenals, pituitary, muscle sample, thyroid and a weighed portion of thymus dissolved in 5 ml of concentrated HNO_3. The acid, after the tissue was digested, was then diluted with distilled water to a measured volume of 50 ml and dip-counted, by a method previously described(2). Aliquots of the injected solutions of I^{131} and P^{32} were prepared and dip-counted in an identical manner as the tissue specimens. All assays were expressed as the percentage of injected activity after allowing for the respective dilutions for assay. To determine the relative ability of a tissue to concentrate radiophosphorus or radioiodine, the percentage of the administered isotope found in an organ was divided by that organ's percentage of body weight. Where only a portion of muscle was assayed, the activity was determined on the basis of one gram and the percentage of body weight of one

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* Aided in part by a grant from the Damon Runyon Memorial Fund.

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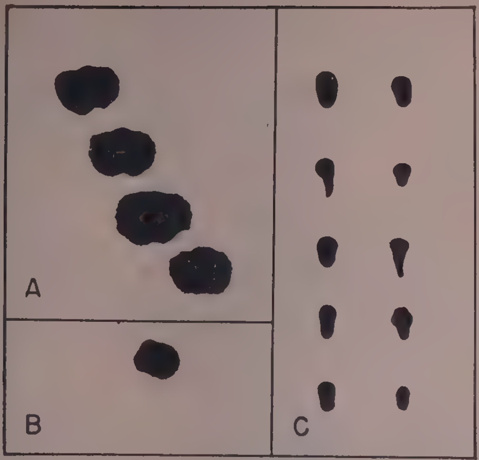

FIG. 1. Positive radioautographs of the thyroid area from: A. normal; B. unilaterally thyroidectomized; C. bilaterally thyroidectomized rats. Excised thyroid area placed directly on double black paper covered no-screen x-ray film for 3 days under light pressure.

gram was used. These ratios were termed the concentration ratios.

The completeness of thyroidectomy was determined by comparing radioautographs including the thyroid area of thyroidectomized animals from this study with radioautographs from non-operated albino rats of the same age and sex similarly treated with radioiodine I^{131} (Fig. 1) adapted from the method of Reinhardt(3).

Results. Young rats. There was observed a profound inhibition of rate of body weight increase 2 weeks following thyroidectomy (Fig. 2). This was seen to inhibit growth to approximately 5 g increase of body weight in 45 days, compared to the control rats which showed an increase of 80 g in the same time interval. The I^{131} concentration ratio was significantly increased in the pituitary (+2650%) of the thyroidectomized rats over the levels found in the control animals, while the differences found in the thymus and adrenal were also significantly higher than those of the control group (Table I). The P^{32} concentration ratios were significantly depressed in the pituitary and adrenals of the thyroidectomized rats compared to the levels in the control animals. Muscle P^{32} concentration ratios were also significantly depressed. An increased pituitary percentage of body weight and a decreased thymus percent body weight was also observed in the thyroidectomized animals.

Adult rats. Little change in body weight of the experimental and control groups was observed (Fig. 2). The concentration ratio of I^{131} was again significantly increased in the pituitary (+250%) in the thyroidectomized animals over the levels found in the control animals. It was found that the thyroidectomized rats showed no significant differences in the concentration ratios of P^{32} compared to the control animals. The adrenals and thymus showed a significantly smaller percentage of body weight in the thyroidectomized animals (Table I).

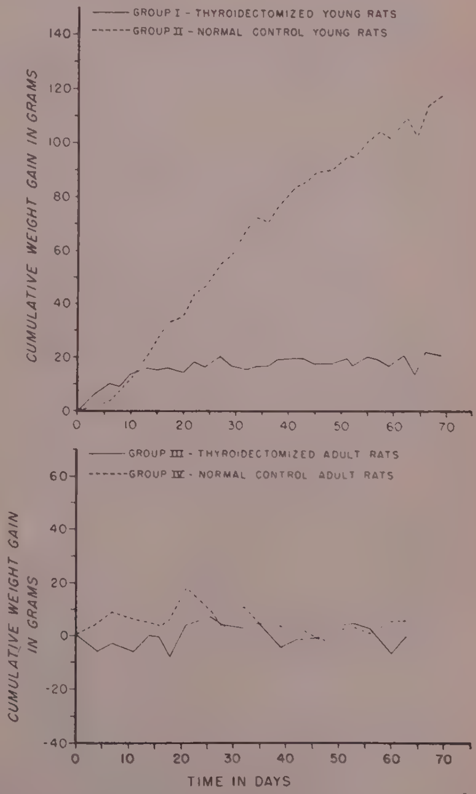

FIG. 2. Cumulative weight gain for 70 days of young (upper graph) and adult (lower graph) male albino rats compared to normal control animals.

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TABLE I. Mean Organ % of Body Wt, I^{131} and P^{32} Concentration Ratios in Thyroidectomized and Control Animals.

	Mean organ % of body weight			
Group	Pituitary	Adrenal	Thymus	Thyroid
I – thyroidect.	.012*	.027	.165†	
II – control	.005‡	.032	.235‡	.013
III – thyroidect.	.007	.020*	.078†	
IV – control	.007	.029	.100	.013

	Mean I^{131} concentration ratios $\left[\frac{\text{Organ's \% total activity}}{\text{Organ's \% body wt}}\right]$				
Group	Pituitary	Adrenal	Thymus	Muscle	Thyroid
I – thyroidect.	1.180*	.506†	.434*	.142	
II – control	.043	.264‡	.233	.129	52.161
III – thyroidect.	.537*	.235	.240	.109	
IV – control	.153	.131	.167	.047	37.064

	Mean P^{32} concentration ratios $\left[\frac{\text{Organ's \% total activity}}{\text{Organ's \% body wt}}\right]$				
Group	Pituitary	Adrenal	Thymus	Muscle	Thyroid
I – thyroidect.	3.741†	3.903*	8.605	.538†	
II – control	6.045	8.232	9.730	1.127	4.867
III – thyroidect.	4.873	5.262	5.236	.788	
IV – control	4.392	8.534	5.171	1.182	4.201

* Significantly different from control values to a probability $<.01$.
† ” ” ” control values to a probability $<.05$.
‡ ” ” ” Group IV control values to a probability $<.01$.

In a comparison between the normal groups of non-operated rats, the mature control animals showed a decreased concentration ratio of I^{131} in the adrenals, compared to the young control animals. The concentration ratios of P^{32} in the adult animals were not significantly different compared to levels seen in the young control animals. However, the older animals showed a significantly greater percentage of body weight in the pituitary and a lower percentage of body weight in the thymus, compared to the younger control animals (Table I).

Discussion and conclusions. It was observed that the effects of thyroidectomy were most noticeable in young rats, and were most pronounced upon the pituitary and the adrenals of these animals. Both the pituitary and adrenals of the young thyroidectomized rat had increased I^{131} concentration ratios and decreased P^{32} concentration ratios. The mature thyroidectomized animals exhibited a significantly increased pituitary I^{131} concentration ratio, but many of the other changes seen in the younger animals were absent or less pronounced.

Greenberg *et al.*(4) concluded that the observed relationships of increased P^{32} uptake in the organs of the hyperthyroid rats and the decreased P^{32} uptake in the organs of the hypothyroid rats, suggests that thyroid activity influences the rate of transfer of phosphorus across cell membranes. Such a conclusion does not offer explanation for the greatly increased iodine (I^{131}) content of the pituitary and adrenal glands of animals reported here.

Summary. 1. Seventy days after thyroidectomy the ability of various organs of young and adult male albino rats to concentrate radioiodine I^{131} and radiophosphorus P^{32} was determined and compared to control animals. 2. The pituitary and adrenals of the young rats showed a markedly increased 3-hour concentration of I^{131} and a decreased 3-hour concentration of P^{32}, significantly different from the control animals. 3. Mature animals exhibited an increased 3-hour concentration of I^{131} in the pituitary, with no significant

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4. Greenberg, D. M., Frankel-Conrat, J., and Glendening, M. B., *Fed. Proc.*, 1947, v6, 1.

differences in the 3-hour concentration of P^{32} from levels seen in the control animals. 4. The deleterious effects of thyroidectomy in the young animals with a high rate of tissue phosphate utilization was evidenced by the sharply decreased P^{32} concentration in the organs of those animals. Explanation for the markedly increased I^{131} concentrations in pituitary and adrenal of young and adult thyroidectomized animals is not readily apparent.

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Dithiol Antagonism and Potentiation of Chemotherapeutic Agents. (19038)

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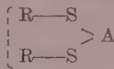
Ehrlich(1) formulated the hypothesis that arsenicals act on pathogenic microorganisms through receptors formed by "conjugated" -SH or -OH groups.† Since then the participation of -SH groups in the mechanism of chemotherapeutic action has been widely studied, particularly after Voegtlin, Dyer and Leonard(2) demonstrated the antagonistic action of monothiols on arsenicals. The literature on this subject and the more recent conception that arsenicals act as thiol-enzyme inhibitors, has been reviewed by Findlay(3), Barron(4), Waters and Stock(5), Peters(6), Stocken and Thompson(7). The last 3 authors were "led . . . to the realization that the more stable form of arsenic with the enzyme must be a ring form in which two sulphur atoms are combined with one arsenic, as follows:  $\left.\begin{matrix}R-S\\R-S\end{matrix}\right> As-$"(6). This idea was confirmed in the successful use of dimercaptopropanol (BAL) as an antidote in lewisite, and more generally, in metal poisoning. We reported(8) that BAL interferes to a much greater extent with the therapeutic action of arsenoxide against *T. equiperdum* than with its toxic effect on the host.‡ The intensive reversal of the therapeutic effect could not be explained alone with the formation of an arsenoxo-BAL compound, since this compound, prepared and studied in various laboratories, maintains a definite therapeutic activity and toxicity(9-12). Searching for an explanation, we assumed that BAL determines the reversal of the therapeutic effect by acting directly on the parasite, thus interfering with the chemotherapeutic process itself, and consequently other drugs than the metal containing ones might be also reversed by the dithiol. We speculated that the antagonistic effect of BAL in the case of arsenicals might have two phases: (a) the dithiol intervenes in the "protection" of the -SH groups of the parasite; (b) it forms an As-BAL compound according to the equilibrium

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* Division of Pharmacology and Chemotherapy, Warner Institute for Therapeutic Research, New York.

1. Ehrlich, P., *Ber. dtsche Chem. Ges.*, 1909, v42, 17.

† Ehrlich was referring explicitly to the dithiol function: "dass vielleicht solche orthoständigen Hydroxyloder Sulphhydrylgruppen durch geeignete Bedingungen befähigt sein können, als Metallreceptoren zu fungieren". Note the analogy with the expression used by modern biochemists (Stocken and Thompson, *Biochem. J.*, 1946, v40, 529): "it seems probable that the arsenic has combined with two -SH groups closely placed in space on the same molecule, to form a ring".

2. Voegtlin, C., Dyer, H. A., and Leonard, C. S., *J. Pharmacol.*, 1925, v25, 297.

3. Findlay, G. M. *Recent advances in chemotherapy*, Vol. I. Churchill, London, 1950.

4. Barron, G. E. S., *Advances in Enzymology*, 1951, v11, 201. Interscience, New York.

5. Waters, L. L., and Stock, Ch., *Science*, 1945, v102, 601.

6. Peters, R. A., *Symp. Soc. Exp. Biol.* III. 36, Cambridge University Press, 1949.

7. Stocken, L. A., and Thompson, R. H. S., *Physiol. Rev.*, 1949, v29, 168.

8. Ercoli, N., and Wilson, W., *J. Pharmacol.*, 1948, v92, 121.

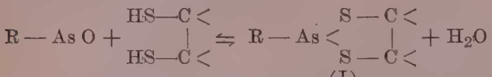
‡ This finds a certain analogy in observations made by Voegtlin on monothiols: "it is more difficult to counteract the toxic effect of As in the higher animals than that exerted on protozoa"(2).

9. Peters, R. A., and Stocken, L. A., *Bioch. J.*, 1947, v41, 53.

10. Friedheim, E. A. H., and Vogel, H. J., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 418.

11. Friedheim, E. A. H., and Berman, R. L., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 180.

12. Sawyers, J. L., Burrows, B., and Maren, H. T., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 194.



$$R{-}AsO + \begin{array}{l} HS{-}C< \\ \quad\;| \\ HS{-}C< \end{array} \rightleftharpoons R{-}As< \begin{array}{l} S{-}C< \\ \quad| \\ S{-}C< \end{array} + H_2O$$

(I)

In the presence of a sufficient excess of dithiol, the "BAL-OXO" compound (I) is stable enough to acquire different characteristics of penetration, fixation and elimination than the original arsenoxide. In fact, according to Peters and Stocken(9) the BAL-OXO compound itself is more toxic for the host than the original arsenical, but it becomes detoxified by additional BAL. According to Eagle and coworkers(13,14) BAL treatment increases noticeably the elimination of the arsenical from the host organism and this is undoubtedly one of the principal mechanisms responsible for detoxification. However, we considered it possible that as far as the antiparasitic effect is concerned, this formation of dithio-arsenate is only an additional factor contributing to the process of antagonism, while the main underlying phenomenon is the "protection" given by BAL to the -SH groups considered involved in the chemotherapeutic mechanism. This direct "protection" of the parasite, whatever its mechanism may be (competitive replacement of -SH groups, or else) should determine an antagonistic effect also in cases where the formation of a compound between therapeutic agent and BAL would not easily occur. This assumption implied that the mechanism of the BAL antagonism is not necessarily of the same nature for the host and for the parasite. Following this hypothesis, we investigated during the years 1948-1951 the influence of the mono- and dithiol functions on the majority of the therapeutic agents in use, in different types of experimental infections.

TABLE I. Effect of BAL on Therapeutic Activity of Fuadin in the *Tr. equiperdum* Infection of Mice.

Fuadin, mg/kg	BAL, mg/kg	No. of mice — Cleared	No. of mice — Cured
8	0	4/10	0/10
8	50	0/5	
16	0	8/10	3/10
16	1.5	1/5	0/5
16	6.25	0/5	
16	25	0/5	
16	2 x 50	0/5	
32	0	15/15*	5/15
32	12.5	5/5†	0/5
32	2 x 50	0/5	

* Within 3 hr.
† " 24 ".

Methods. The technics used to obtain the *T. equiperdum*(8), *Borrelia novyi*(15) and *duttoni*(16), *Streptococcus hemolyticus* and *E. coli*(17) infections of mice were described. The minimal effective and average curative doses have been precisely determined both in the protozoan(16) and bacterial infections (17). Dermal syphilis of the rabbit was induced by injecting intradermally 0.25 cc of a testicular suspension containing 5 *Tr. pallidum* per dark field: lesions developed within 14 to 21 days. The influence of BAL on the chemotherapeutic action has been established as follows: (a) in protozoan infections, by the influence of various BAL doses on the effectiveness of active dosages of the drug on the parasitemia following treatment and the rate of cure; (b) in bacterial infections, by the mortality rate change induced by BAL on protective ($CD_{50}$and CD_{90}) drug doses. From all dead animals heart cultures were made, and the bacteriological findings were considered in interpreting the results. BAL was injected subcutaneously in oil at the same time when therapeutic treatment was given; if a second injection of BAL was made, this was done

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13. Eagle, H., Magnuson, H. J., and Fleischman, R., *J. Clin. Invest.*, 1946, v25, 451.

14. Wexler, J., Eagle, H., Tatum, H. J., Magnuson, H. J. and Watson, E. B., *J. Clin. Invest.*, 1946, v25, 467.

15. Ercoli, N., and Lafferty, L. C., PROC. SOC. EXP. BIOL. AND MED., 1944, v57, 4.

16. Carminati, G. M., and Ercoli, N., *Boll. Ist. Sieroter. Milanese*, 1951, v30, 97.

17. Carminati, G. M., *Boll. Ist. Sieroter. Milanese*, 1951, v30, 349.

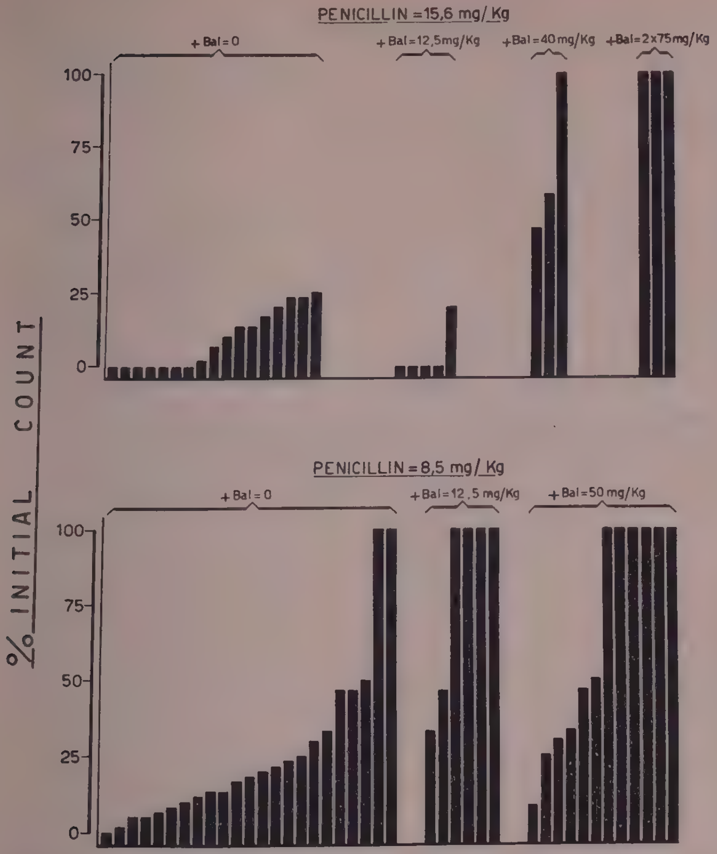

FIG. 1. Antagonistic effect of BAL on therapeutic activity of penicillin in *B. novyi* infection. (Each column represents one mouse.)

after 2 to 4 hours, the approximate duration of the dithiol effect. If not stated otherwise, drugs were administered subcutaneously. Freshly opened ampuls of oily BAL solutions, diluted as required, in oil, just before treatment are recommended.

Results. 1. *Trypanosoma equiperdum infection.* Therapeutic doses of Acriflavine (2.8 diamino-acridinium-10-metho-chloride) (4 x 25 mg/kg), Stilbamidine (4,4-diamidinostilbene) (25 mg/kg), and Naphuride (Trisodium salt of 8,8′ [3″,3‴ -ureylenebis(3″″-bensamido-4″″ methylbensamido)] bis 1,3,5-naphthalene-trisulfonic acid) (16.5 mg/kg) were not influenced in their effectiveness by BAL doses as high as 2x50 mg/kg. Fuadin (Antimony-pyrochatecol-sodium disulfonate) doses which cause disappearance of the trypanosomes within 24 hours, without permanent cure, (8-16 mg/kg) have been inhibited by 6.0, and occasionally by 1.5 mg/kg BAL. A higher Fuadin dose, 32 mg/kg, which causes complete disappearance of the parasites within 3 hours and sterilizes 30% of the mice, has been completely inhibited by 2 x 50 mg/kg BAL. With lower BAL doses, 12.5 mg/kg, the disappearance of the parasites was delayed from 3 to 20 hours and the survival period was shortened from 18 to 10 days. In general, Fuadin reacted to BAL in a manner similar to that reported for oxophenarsine(8); with a progressive increase of the BAL dosage the curative effect decreased, as revealed by the length of the relapse period and the percentage of relapsing animals (Table I).

2. *Borrelia novyi and duttoni infections.*

TABLE II. Influence of BAL and Glutathione on Therapeutic Activity of Penicillin G (50 mg/kg) in *B. duttoni* Infection.

Treatment	No. spirochetes/100 dark fields after—				
	Before	2½ hr	4½ hr	21 hr	42 hr
Penicillin alone	300	12	0	4	0
	450	4	0	2	0
	260	8	0	4	0
	300	0	0	2	0
Penicillin + BAL, 62.5 mg/kg	300	350	100	80	800
	200	400	170	100	1000
	150	350	100	60	550
	200	500	180	100	1000
Penicillin + glutathione, 150 mg/kg	200	2	0	0	0
	270	4	0	0	0
	200	0	0	0	0
	170	4	0	0	0

TABLE III. BAL Antagonism of Bacitracin in *B. duttoni* Infection of Mice.

Bacitracin, mg/kg	BAL, mg/kg	No. spirochetes/100 dark fields after—				
		Before	3 hr	6 hr	24 hr	48 hr
3	0	200	72	0	400	
		170	48	0	30	
		200	32	12	50	
3	50	200	200	600	2000	
		200	180	400	1200	
		200	216	200	1500	
10	0	300	8		8	100
		80	0		0	40
		250	0		0	100
		350	0		0	0
10	2 × 50	200	100		600	3000
		200	130		1300	2000
		180	45		1000	500
		300	100		32	3000
20	0	250	0		0	0
		280	0		0	0
20	2 × 50	150	80		50	1000
		300	350		400	1500

BAL had no influence on therapeutic doses of streptomycin (75-160 mg/kg), aureomycin (2-8 mg/kg) and terramycin (4-10 mg/kg). BAL doses of 4-8 mg/kg inhibited completely the clearing effect of 10 mg/kg oxophenarsine. Penicillin was completely or partially inhibited[§] according to the penicillin/BAL dose relationship. The effect of a penicillin dose (30-50 mg/kg) which results in a rapid (2-3 hours) disappearance of the spirochetes has been practically destroyed by a single dose of 50-65 mg/kg BAL. Typical experiments showing the reversal of the antispirochetal action of penicillin by BAL are presented in Fig. 1 (*B. novyi*) and Table II (*B. duttoni*). The inhibition of the therapeutic action could not be obtained if BAL treatment was delayed by 2 hours or more. Multiples (20-50 mg/kg) of the therapeutic bacitracin dose (7-13 mg/kg) were completely inhibited by BAL as shown in the experiment presented in Table III. Chloromycetin doses above the minimal effective one (35 mg/kg) up to 80 mg/kg were inhibited by a single dose of 60 mg/kg BAL (Table IV). Higher doses of chloromycetin (100-150 mg/kg) were not influenced by 2 x 40 or 50 mg/kg BAL. With penicillin and bacitracin the BAL reversal was very regular and the doses required were the same in the *B. novyi* and *duttoni* infections (used respectively in the American laboratory and abroad) at the point that we

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[§] This observation was reported by Ercoli, Whitehead and Schwarz at the 48th Gen. Meeting Soc. Am. Bact., Minneapolis, Minn., 1948.

TABLE IV. BAL Antagonism of Chloromycetin in *B. duttoni* Infection of Mice.

Date	Chloromycetin, mg/kg	BAL, mg/kg	No. spirochetes/100 dark fields after			
			Before	3 hr	21 hr	44 hr
XII/20/50	30	0	300	32	12	—
			500	12	0	—
	30	2 × 60	500	300	72	—
	”	”	900	790	800	—
I/3/51	45	0	300	4	12	0
			500	20	0	300
			400	8	32	200
			400	12	8	500
			200	40	80	80
	45	75	800	1500	1000	2000
			600	1800	2500	2200
			400	600	200	2000
			600	600	1000	5000
			700	500	1200	2000
XI/27/50	70	0	300	0	0	0
			300	0	0	0
			400	0	24	0
	70	60	300	4	80	100
			500	48	30	80
			200	32	60	200
X/30/50	80	0	200	0	0	0
			400	0	0	0
	80	2 × 60	400	32	8	1500
			300	200	16	100

TABLE V. BAL Potentiation of Subtilin in *B. duttoni* Infection of Mice.
(Exp. 70/E–III/3/51.)

Subtilin, mg/kg	BAL, mg/kg	No. spirochetes/100 dark fields after				
		Before	3 hr	20 hr	30 hr	48 hr
Controls		{ 400	600	1000	1400	1800
		500	800	1200	1600	2000
8	0	200	72	12	48	200
		300	100	60	300	800
		150	60	100	100	19
		300	100	800	800	1000
		200	22	300	400	600
8	50	300	0	72	8	1500
		300	0	64	24	600
		400	0	8	20	500
		300	0	4	12	200
		400	8	0	4	800
16	0	300	32	200	40	500
		400	200	500	1000	2300
		300	100	400	80	200
		400	0	300	40	10
		300	40	500	1200	2000
16	50	1500	4	0	0	0
		400	0	0	0	0
		300	0	0	0	0
		300	0	0	4	0
		400	0	0	0	0

have used this phenomenon for a rapid titration of BAL preparations; with chloromycetin the reversal did not reach the same degree of regularity. In contrast to the effect of BAL on the antispirochetal activity of the above antibiotics, subtilin (8-16 mg/kg) in some experiments was potentiated not only by the dithiol, but also by monothiol cysteine (150 mg/kg). However, for reasons which we cannot explain, the response of subtilin to

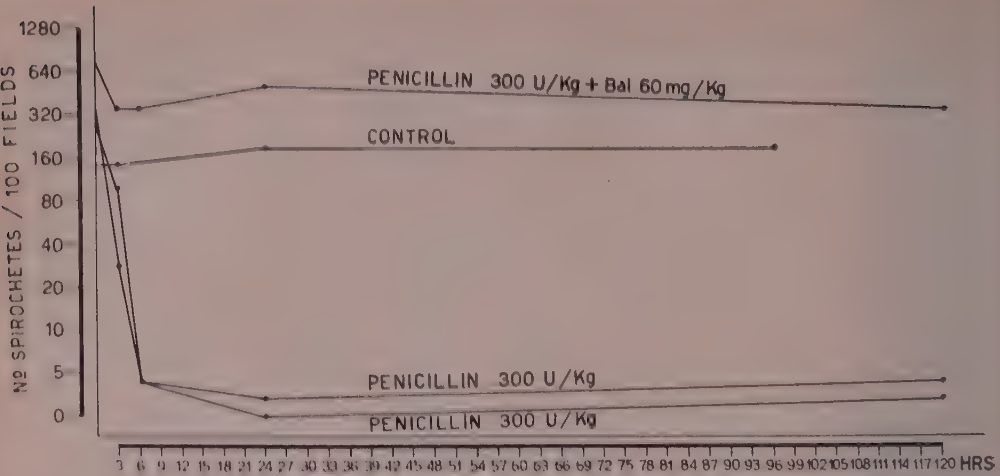

FIG. 2. BAL inhibition of antispirochetal action of penicillin in dermal syphilis.

the thiols is not constant. So far, potentiation with cysteine and BAL has been obtained in 2 out of 6 experiments while in the other 4 experiments the effect of subtilin has not been influenced by the thiols. One of the 2 practically identical experiments in which the subtilin potentiation appeared is presented in Table V. Cysteine had no effect on the antibiotic inhibited by BAL: in doses of 150 mg/kg it did not influence the activity of bacitracin, chloromycetin (and aureomycin); cysteine and glutathione doses as high as 500 mg/kg were without any effect on the therapeutic activity of penicillin (8-32 mg/kg). Cysteine doses of 150 mg/kg did not influence the therapeutic activity of 5 mg/kg oxophenarsine hydrochloride; but the same dose had a slight effect on a lower dose of arsenical: 2 out of 5 mice treated with 2.5 mg/kg oxophenarsine + cysteine were not cleared, while in the control groups this dose of arsenical gives in all cases complete (or 90%) reduction of the spirochetes within 24 hours. The therapeutic effect of gold thiomalate (Myochrysin)—which in doses of 25 mg/kg clears the blood stream within 24 hours and in doses of 75 mg/kg is curative—has been completely inhibited by 2 x 60 mg/kg BAL.

3. *Dermal syphilis of rabbit.* We controlled on *Tr. pallidum* the results obtained with penicillin in relapsing fever infections. Fig. 2 shows that in this infection too the therapeutic action of penicillin is reversed by BAL.

4. *Streptococcal infection.* The average protective dose of penicillin, 1-1.3 mg/kg (*i.e.* about 1/20th of the clearing dose in Borrelia infections) was not inhibited by BAL doses as high as 2 x 60 mg/kg. The average protective doses of the other antibiotics tested, namely subtilin (1.25-2.5 mg kg), aureomycin (10 mg/kg), bacitracin (6 x 20 or single doses of 60-120 mg/kg), chloromycetin (150 mg/kg), streptomycin (140 mg/kg), terramycin (10 mg/kg) remained unaffected even by the highest tolerated BAL doses. The only anti-streptococcal agent inhibited by BAL is Myochrysin (Table VI). The doses required to inhibit the effect of 50-75 mg/kg Myochrysin is 50 mg/kg BAL, while a dose of 100 mg/kg requires for inhibition 2 treatments with 50 mg/kg BAL.

5. *E. coli infection.* BAL had no effect on streptomycin (6-40 mg/kg) terramycin (12-20 mg/kg), chloromycetin (160-230 mg/kg) and sulfadiazine (10-20 mg/kg). Aureomycin (30-40 mg/kg) given simultaneously with BAL (2 x 40 or 60 mg/kg) gave a larger number of deaths. This was found to be due to a combined toxic effect, and not to a decrease of therapeutic action: there was no difference in effectiveness between the series with and without BAL, when the animals which died with negative heart cultures were taken into account.

The effect of penicillin in this infection was significantly potentiated by BAL. In the

TABLE VI. Effect of BAL on Therapeutic Activity of Myochrysin in *Str. hem.* Infection of Mice.

Myochrysin, mg/kg	BAL, mg/kg	No. mice	% survivors
50-75	0	20	85
50-75	50-60	20	15
100	0	50	72
100	2 × 30	10	60
100	50-60	30	60
100	2 × 50	20	5

TABLE VII. Influence of BAL on Therapeutic Activity of Penicillin in *E. coli* Infection.

Penicillin, mg/kg	BAL, mg/kg	Alive/total mice	% survivors + sterilized
100	0	0/10	0
100	2 × 50	4/10	40
150-160	0	9/25	36
150-160	2 × 40-50	14/25	56
200	0	18/50	36
200	2 × 50 or 1 × 60	28/60*	66.6

* + 12 mice died with negative heart cultures.

TABLE VIII. Influence of BAL on Blood Levels of Mice Treated with 100 mg/kg Penicillin (Subcutaneously).

Date	BAL, mg/kg	μ/cc* . . . hr after treatment 1½	3½	5½
V/23/51	0	7.9	.062	0
	62.5	69.5	.5	.031
V/28/51	0	3.9	.062	0
	40	16	.125	.062

* Average of 4 determinations.

group of mice treated with penicillin alone (100-200 mg/kg) 31.7% survived (27/85), in the BAL group 48.4% (46/95). However, in the BAL group 12 animals died with negative heart cultures, which brings the number of the sterilized animals to 61% (58/95). Thus, in spite a certain incidence of combined toxic effect, the survival rate was higher in the BAL treated series and the percentage of sterilized animals was about double (Table VII).

6. *In vitro experiments.* We considered it possible that the BAL potentiation of penicillin in the *E. coli* infection depends on a "pharmacological" and not on a "chemotherapeutic" mechanism. Therefore we decided to examine *in vitro* the antibacterial action of the blood of mice treated at high penicillin dose ranges, as required in the *E. coli* infection. We selected for these experiments *Strept. hem.* C203, an organism which by itself maintains its penicillin sensitivity under BAL treatment. Using the technic recommended by Rammelkamp(18), we found that the antibacterial action of the blood of mice treated with high penicillin dosages is increased by BAL treatment, both, as initial peak and duration of detectable blood concentration (Table VIII). This finding adds to the significance of the antagonism noted in the spirochetal infections: the therapeutic effect is inhibited in spite of the higher penicillin blood levels which may follow BAL treatment. This retention of penicillin is perhaps in relation to the antidiuretic and antichlorouretic effect of BAL reported by Earle and Berliner (19), and probably with the accumulation of the thiol in the kidney, as described for the radioactive material by Peters *et al.*(20).

Discussion. Summarizing our experimental results (Table IX), the known antiprotozoan agents can be divided in 2 classes according whether they are inhibited by BAL or not. *BAL reversible* are: the metal containing compounds (As, Au, Sb), penicillin, bacitracin and chloromycetin; *non BAL reversible* remain: streptomycin, aureomycin, terramycin, naphuride, stilbamidine, acriflavine. Subtilin in most cases was not affected, but occasionally was potentiated both by cysteine and BAL. It should be acceptable that a common inhibitor of the same physiological (antispirochetal) effect depends on an identical mechanism, or receptor system. Thus, such widely different chemical structures as metallic compounds, penicillin, bacitracin (a polypeptide) and chloramphenicol would have a similar antispirochetal mechanism. This inhibiting effect of the dithiol is specific under various aspects. First, the monothiols (cysteine and glutathione) do not possess the

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18. Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, v51, 95.

19. Earle, D. P., and Berliner, R. W., *Am. J. Physiol.*, 1947, v151, 215.

20. Peters, R. A., Spray, G. H., Stocken, L. A., Collie, C. H., Grace, M. A., and Wheatley, G. A., *Biochem. J.*, 1947, v41, 370.

TABLE IX. Effect of Mono and Dithiols on *in vivo* Activity of Chemotherapeutic Agents.

Drug	Effect of BAL on activity in . . . infection			
	Spirochetal	Trypanosomal	*E. coli*	*Strept. haem.*
Arsenoxide	Antagonized*	Antagonized		
Fuadin		,, ‖		
Acriflavine		None		
Stilbamidine		,,		
Naphuride		,,		
Bacitracin	,, †			None†
Penicillin	,, ‡		Potentiated	,, †
Chloromycetin	,, †		None	,,
Subtilin	Occasional potentiation§			None†
Aureomycin	None†		,,	,, †
Terramycin	,,		,,	,,
Streptomycin	,,		,,	,,
Sulfadiazine			,,	,,
Myochrysine	Antagonized			Antagonized

* Cysteine: very slight antagonism. † Cysteine had no effect. ‡ Cysteine and glutathione had no effect. § Same effect with cysteine. ‖ Toxicity for the host increased.

antagonistic activity of BAL. A similar specificity of the reversing effect of the dithiol has been reported for a number of enzymatic inhibitors: the inactivation of pyruvate oxidase by lewisite(21) and by therapeutic arsenicals (22) is reversed by BAL and not by monothiols. In the case of invertase inhibition by $HgCl_2$, BAL reverses, while cysteine even potentiates the effect of $HgCl_2$(23). This analogy of specificity suggests that an enzyme system of similar behavior ("dithiol enzyme") might be involved in the chemism of the therapeutic action of the *BAL-reversible* drugs. However, in view of the extremely complex and multiple participation of thiol functions in biological systems(4) and of the fact that BAL has other chemical properties (strong reducing agent), we do not feel authorized to express on the basis of the present experimental material a definite opinion on the intimate nature of this mechanism. We do feel though that our observations can be utilized as a base to explore with the methods of modern biochemistry and enzymology the mechanism involved in the antiprotozoan (antispirochetal) action of these drugs.

Another manifestation of specificity of the dithiol antagonism is that the drugs inhibited in the spirochetal infection are not affected by BAL in the bacterial infections. This proves that a chemical inactivation (of the drug by BAL) is not at play; otherwise the small—1 mg/kg—penicillin doses used in the coccal infection would have been totally inhibited.

At higher penicillin dose ranges it was found that BAL—probably due to a competitive renal elimination—increases the blood level and therefore the antagonism is higher than it appears considering the dose of penicillin injected. In fact, the increased penicillin levels cause the impression of a potentiation in the *E. coli* infection.

The lack of antagonistic effect of BAL in the bacterial infections proves that the therapeutic mechanisms responsible for antispirochetal and antibacterial actions of penicillin, chloromycetin, bacitracin are certainly not identical. The only drug inhibited by BAL in both, spirochetal and streptococcal infections is Myochrysin, which may be presumed, therefore, to act by the same mechanism in the two infections.

Interestingly, Fuadin which according to the finding of Van Dyke and coworkers(24) becomes more toxic by simultaneous BAL treatment (as confirmed repeatedly on mice

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in the present study) has been easily inhibited as a therapeutic agent in the trypanosomal infection. This forms another evidence for our initial hypothesis that drug toxicity and its inhibition (by BAL) in the case of the host and the parasite are not necessarily bound to the same mechanism. A similar specific inhibition of the trypanocidal and not of the toxic effect has been described by Schleyer and Schnitzer(25), who reported the antagonistic action of methyl-p-hydroxybenzoate against acriflavine and arsenicals.

With the experiments presented the participation of the dithiol function is extended to such widely different processes as, for instance, the antispirochetal activity of antibiotics, the diabetogenic action of alloxan (26), the plant-growth inhibiting effect of cumarin and protoanemonin(27), the detoxification of lewisite and metallic compounds in mammals, protozoans(7), and viruses(28), the activation of papain(29) and cathepsin (30) and their antagonization by carcinogenic hydrocarbons(30,31).

Summary and conclusions. (1) In experimental spirochetal infections BAL reverses the therapeutic activity of penicillin, bacitracin, chloromycetin, oxophenarsine and gold compounds (Myochrysin), while it has no influence on streptomycin, terramycin and aureomycin. (In most cases subtilin has not been affected, occasionally it has been potentiated by BAL and by cysteine). (2) Monothiols (cysteine and glutathione) do not induce the reversal obtained with BAL. (3) In trypanosomal infection BAL reverses the therapeutic activity of arsenicals and Fuadin, while it has no influence on Naphuride, Stilbamidine and Acriflavine. The toxicity of Fuadin for the host(24) is noticeably increased by BAL treatment. (4) In experimental streptococcal infection none of these antibiotics has been influenced by BAL (or monothiols), except Myochrysin, which has been inhibited by BAL. (5) In the *E. coli* infection only penicillin was influenced by BAL: its activity was *increased*. This apparent potentiation was found to be due to a blood level increase determined by BAL with higher penicillin doses. (6) It is concluded that a number of chemically different therapeutic agents (penicillin, bacitracin, chloromycetin, Au, Sb, As compounds) act by a similar mechanism involving the dithiol function in the protozoan infection, while their activity is of a different nature in bacterial infections (probably with the exception of the gold compound). It is considered that BAL inhibition of the drug does not take place with an identical mechanism for the host and the parasite. The specific BAL reversal in the spirochetal infection (and not in others) proves that this is due to a biological effect and not to a chemical inactivation of the drug.

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Comparison of Citrated and Oxalated Plasma in the Photoelectric Determination of Prothrombin Time. (19039)

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In a previous report a method for the photoelectric determination of prothrombin time was described(1) based upon the observation that the optical density of plasma increases sharply at the instant of clotting, thus producing a movement of the galvanometer lightbeam to the left. The original experiments were carried out with oxalated plasma. However, subsequently a comparison of the photoelectric mechanisms involved in the clotting of oxalated and citrated plasma revealed a more distinct "endpoint" when the latter was used. In order to investigate this fact and to properly evaluate the difference between prothrombin times obtained with oxalated and citrated plasma, the present study was undertaken.

Material and methods. One hundred specimens of venous blood were collected from normal individuals. Each specimen was distributed into two tubes in a ratio of nine volumes of blood to one volume of 0.1 M solution of sodium citrate and 0.1 M solution of sodium oxalate, respectively. The prothrombin time was determined in triplicate on each specimen of plasma according to the photoelectric modification of the one-stage Quick method(1). In another series of 50 specimens of blood of normal subjects, the same procedure was employed using, however, the conventional one-stage Quick method(2). Prothrombin times of 2 series of plasma dilutions were established photoelectrically, utilizing both citrated and oxalated plasma. The

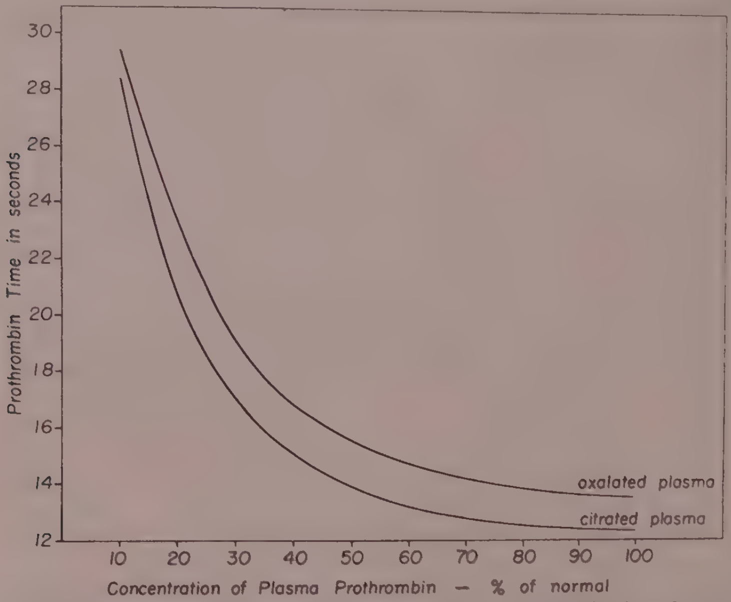

FIG. 1. Comparison of the concentration of prothrombin in serial dilutions between normal citrated and normal oxalated plasma by the photoelectric method.

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TABLE I. Comparison of Mean Values, Range and Stand. Dev. of Prothrombin Times Obtained with Citrated and Oxalated Plasma Using the One-Stage Quick Method and the Photoelectric Method.

	One-stage Quick method		Photoelectric method	
	Mean prothrombin time 50 normal individuals	Stand. dev.,* (sec)	Mean prothrombin time 100 normal individuals	Stand. dev.,* (sec)
Citrated plasma	13.1 sec; range 12.4 to 15.8; 150 determinations	.39	12.6 sec; range 11.4 to 14.6; 300 determinations	.15
Oxalated plasma	14.5 sec; range 13.2 to 16.5; 150 determinations	.43	13.8 sec; range 12.5 to 15.1; 300 determinations	.19

* Stand. dev. obtained by applying formula $\sigma = \sqrt{\frac{\Sigma d^2}{N}}$.

results were plotted against the respective concentrations (Fig. 1).

Results. The prothrombin times of 100 normal individuals as determined with the photoelectric method using citrated plasma ranged from 11.4 to 14.6 seconds with a mean value of 12.6 seconds and a standard deviation of 0.15 second (Table I). When oxalated plasma was used the prothrombin times varied from 12.5 to 15.1 seconds with a mean value of 13.8 seconds and a standard deviation of 0.19 second. The prothrombin times of 50 normal individuals as determined with the conventional one-stage Quick method ranged from 12.4 to 15.8 seconds with a mean value of 13.1 seconds, and a standard deviation of 0.39 second using citrated plasma. The prothrombin times varied from 13.2 to 16.5 seconds, with a mean value of 14.5 seconds, and a standard deviation of 0.43 second when oxalated plasma was employed.

The plasma dilution curve obtained with citrated plasma showed uniformly lower prothrombin times than those with oxalated plasma, the difference being slightly more than one second at each plasma concentration (Fig. 1).

Discussion. This study demonstrates the advantage of the use of citrated plasma in the photoelectric determination of prothrombin time. It was observed that the "endpoint" of clotting is thus more distinctly established as compared with oxalated plasma. Moreover, a greater accuracy has been achieved, as evidenced by the standard deviation of only 0.15 second. When oxalated plasma is used in the photoelectric determination of prothrombin time, the standard deviation has been found to be 0.19 second. This approximates the value previously reported with this method (1). The disadvantage of oxalated plasma, it is believed, is probably due to the fact that upon addition of calcium chloride, a precipitate of calcium oxalate is formed which causes the galvanometer lightbeam of the spectrophotometer to move slowly to the left before the actual endpoint is reached. On the other hand, no precipitate but a soluble complex of calcium citrate(3) is formed upon the addition of calcium chloride if citrated plasma is used. Correspondingly, at the onset of clotting, the swing of the lightbeam indicating the "endpoint" is therefore more distinct with citrated than with oxalated plasma.

It has been known that sodium citrate very quickly removes ionic calcium, whereas the action of sodium oxalate is relatively slow(4). After recalcification of citrated plasma the prothrombin time may be prolonged if lower concentrations of calcium are used as compared with the values obtained with oxalated plasma(5). The addition of .01 M solution of calcium chloride or higher concentrations of calcium chloride to citrated plasma, however, yields an optimal prothrombin time according to Quick(5). In the present study recalcification was performed with .02 M solution of calcium chloride. The resulting prothrombin

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times seem to warrant the conclusion that citrated plasma yields optimal values as compared with oxalated plasma. This, in conjunction with the aforementioned factors would make citrated plasma more suitable for the photoelectric determination of prothrombin time than oxalated plasma.

Summary. (1) In a series of 600 photoelectric determinations of the prothrombin time of 100 normal individuals, the mean value for citrated plasma was 12.6 seconds as compared to 13.8 seconds for oxalated plasma. (2) The standard deviation was 0.15 second for citrated as compared to 0.19 second for oxalated plasma. (3) The onset of clotting is more easily determined photoelectrically with citrated than with oxalated plasma. This is evidenced by a more distinct swing of the galvanometer lightbeam because no precipitate but a soluble complex of calcium citrate is formed upon addition of calcium chloride.

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Blood, Plasma and "Globulin" Space of Guinea Pigs Determined with I^{131} Rabbit Globulin. (19040)

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(With the technical assistance of Patricia Miller and Roswell Reed.)

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Iodine^{131} trace-iodinated-proteins have been used to determine vascular volumes in the dog(1), mouse(2) and man(3). In the course of studies of I^{131} trace-iodinated-rabbit antibody(4) it was necessary to determine the volume of guinea pig plasma in which this protein was diluted after intravenous administration. Since blood and plasma volumes have not been published for the guinea pig in the recent literature(5-7) except for a report of the plasma volume of 4 guinea pigs using T-1824(8), it was felt desirable to present the data.

TABLE I. Data on I^{131} Rabbit Globulin.

Amt protein nitrogen injected (mg)	12.63
,, I^{131} injected (μc)	3.94
,, protein bound iodine injected (μg)	200
No. iodine atoms per protein molecule	3.2
% activity not precipitable with trichloracetic acid	.51
Volume globulin injected (cc)	2.5

Methods. The globulin studied was obtained from immune rabbit serum and iodinated by a method described previously(9). Table I presents the composition and pertinent data of the preparation used in this study. The guinea pigs received the I^{131} rabbit globulin intravenously, and blood was obtained from the ear with a 20 mm^3 Sahli pipette at the times indicated in Table II. The blood was immediately delivered into an ointment tin sample dish lined with lens

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* Postdoctoral Research Fellow, National Cancer Institute.

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TABLE II. 8-15 Min Dilution Vol of I^{131} Rabbit Globulin in the Guinea Pig. All Males.

Guinea pig No.	Wt, g	Time after inj for sample, min	Blood vol, cc/100 g body wt	Plasma vol, cc/100 g body wt
15	464	12	7.32	3.83
16	525	8	7.70	4.03
17	476*	9	7.28	3.81
18	479*	13	8.52	4.46
19	493*	11	7.32	3.83
20	490*	13	7.72	4.05
21	414	14	7.14	3.74
22	461	9	7.90	4.14
23	441	10	7.44	3.90
24	483*	13	6.72	3.52
25	505	12	6.70	3.51
26	543	15	9.24	4.84
27	529	8	6.92	3.62
Mean			7.53 ± .71†	3.94 ± .37†

* Only these animals were used for determination of the "globulin space."

† Stand. error of the mean.

paper, and the pipette washed with 1:10,000 heparin 2 to 3 times until free of blood, all washings being delivered into the sample dish. The samples were dried with an infra-red lamp and the beta activity determined with an end window Geiger-Mueller tube (about 2.0 mg/cm² mica window). No mass absorption correction was needed since the mass of the samples was less than 3×10^{-3} mg/cm². An aliquot of the total amount of radioactivity administered was counted simultaneously with the blood sample. The 8 to 15-minute dilution volume of the labelled foreign protein in whole blood was then determined. The plasma volume was calculated in terms of an average hematocrit of 47.6, which was obtained on 10 male guinea pigs weighing from 445 to 841 g. The hematocrits were determined on heparinized blood in a Wintrobe hematocrit tube which was centrifuged in an International clinical model centrifuge for 30 minutes at 3,800 rpm ($G_{max} = 1200$). No correction was employed for entrapped plasma.

Results. Table II presents the blood and plasma volumes per 100 g body weight in 13 guinea pigs. The blood volume was determined from the relationship.

$V = a/A - v$ where,

a = activity injected,

A = activity per unit vol. blood at time sample was obtained, and

v = vol. inj.

The 8 to 15-minute dilution volume in whole blood was found to be 7.53 ± 0.71 cc† per 100 g body weight, and the plasma volume was 3.94 ± 0.37 cc† per 100 g body weight. The direct experimentally determined value in these data is the blood volume, whereas the plasma volume is a derived value in terms of the hematocrit obtained in this experiment. Consequently the "true" plasma volume will differ from the value reported, depending on the deviation of the centrifuged hematocrit from the "true" hematocrit. Since there is a great deal of controversy concerning the correction of the centrifuged hematocrit(10,11) no attempt has been made to correct our centrifuged hematocrit.

Discussion. Fig. 1 is a plot of the per cent of the administered radioactivity remaining per ml serum as a function of time over a period of 5 days in 5 guinea pigs with an average weight of 480 g. Analysis of this curve reveals 2 principal components, an initial rapid decrease of activity followed by a slow single exponential rate of degradation of the rabbit globulin in the guinea pig (4). This interpretation is based on the fact that the radioactivity remaining in these animals, as obtained from excretion data, follows a single exponential function with about the same half life as the slower component in the serum activity-time curve. The initial rapid decrease of activity in the serum in the first 20 hours therefore represents either a dilution of the labelled foreign protein into a space larger than the 8 to 15-minute dilution volume or an initial pooling of the labelled protein in some tissue. Since the activity in the thyroids of animals sacrificed at 4 hours was less than 0.06% and the highest tissue concentration attained was 0.48% in the kidney(12), this initial rapid decrease in serum activity cannot be accounted for by I^{131} leaving the blood and

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† Standard error of the mean.

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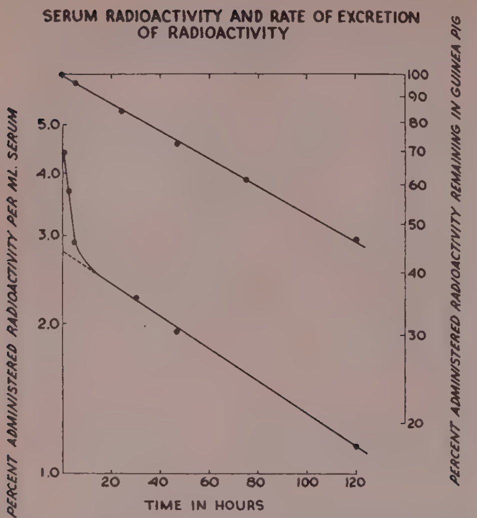

FIG. 1. Serum radioactivity and rate of excretion of radioactivity in 5 guinea pigs following intravenous administration of I^{131} trace iodinated rabbit globulin.

being pooled in the thyroid or in other tissues. Kreiger *et al.*(13) have shown that I^{131} labelled protein slowly enters the thoracic duct lymph and has correlated this initial rapid rate of disappearance of activity with the dilution of the labelled protein in the lymph space. In view of these findings extrapolation of the slower rate to zero time should yield the total volume into which the protein mixes. This calculation, based on a composite curve of 5 guinea pigs, yields a final protein dilution volume of 7.58 cc/100 g body weight, if the concentration of the foreign protein in this larger volume is the same as in the blood. The estimated value gives a minimum volume, since this volume is inversely proportional to the concentration of protein found in this fluid. The 8 to 15-minute dilution volume (peripheral plasma volume) therefore constitutes a maximum value of 59% of the total body fluid volume into which the protein is diluted. The "globulin space" is probably made up of an undetermined number of compartments, which include liver lymph, thoracic duct lymph and other extravascular globulin spaces, since the initial rapid decrease in the serum activity curve is not a single exponential rate.

Summary. (1) The blood and plasma volumes of guinea pigs were determined by the dilution of I^{131} trace iodinated rabbit globulin. (2) The average 8 to 15-minute dilution blood volume was found to be 7.53 ± 0.71 cc[†] per 100 g body weight, and the average plasma volume was 3.94 ± 0.37 cc[†] per 100 g body weight. (3) The labelled protein was found to mix slowly over a period of 20 hours into a larger body fluid volume which was found to have a minimum value of 7.58 cc/100 g body weight, which includes the peripheral plasma volume and a number of undetermined compartments.

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Shortening of Uterine Muscle at Different Temperatures. (19041)

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The experiments here reported show that the amount of shortening of uterine muscle when contracting *in vitro* is a function of temperature. There are no previous reports on this question as regards uterine muscle. On smooth muscle in general there is only one report(1), on the dog's retractor penis, and this deals with only a narrow temperature range (16°-35°C). The literature on the relation between temperature and shortening of

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skeletal muscle is summarized by Fenn(2) and by Hajdu and O'Sullivan(3). The question has an important bearing upon the thermodynamic concept of muscle contraction, recently emphasized by Szent-Györgyi, which has led to new experimental approaches. Varga, in association with Laki(4), found that the degree of shortening of washed frog and rabbit muscle, elicited by adenosine triphosphate, is a function of temperature. The same was found true of the contractile substance of skeletal muscle in a state still further removed from the living condition, *i.e.*, threads of actomyosin extracted from the muscle. Szent-Györgyi(5) made the assumption that the contractile matter is built of small units which obey an equilibrium reaction of the first order, and that the shortening of the whole muscle is the expression of the shortening of these units, which have been called autons. The autons are supposed to act in all-or-none fashion. Under these assumptions, the length of the muscle indicates the relative number of relaxed and contracted autons, which is the equilibrium constant (K) of the reaction. Since equilibrium reactions are directly influenced by temperature, the graph of log K plotted against 1/T should give a straight line.

These assumptions, and the general justifiability of applying thermodynamics to muscle, have been criticized by Hill(6) and restated by Szent-Györgyi(4). Recently Hajdu(7), Hajdu and O'Sullivan(3), and Varga(8) have extended their observations to living muscle of the frog and rat and to glycerol-treated rabbit muscle. They find that both the tension and the shortening developed by these muscle preparations depend quantitatively upon temperature. Although the present authors are not prepared to discuss the applicability of thermodynamics to muscle contractions, the present experiments are presented as relevant to the discussion.

Strips of uterine muscle were prepared from rabbits in estrus. Immediately after mating the animal was anesthetized with intravenous Nembutal. The uterine horns were quickly removed and immersed in pre-oxygenated modified Locke solution at 0°C at pH 7.0 (NaH_2PO_4 buffer). Identical segments 2.5 cm long, 0.5 cm wide and weighing about 2 g were cut from the uterine horn, and placed in 50 ml tubes of the recording apparatus. The tubes were filled with the modified Locke solution, which was kept oxygenated at 2.0 vol. %. The bath temperature was 35°C. A constant load of 3 g (including friction) was applied. Two strips were used simultaneously as mutual controls. The length of the writing lever and other kymographic details were kept constant. For a detailed description of the method see Csapo and Corner(9). Electrical stimulation was provided by 20 volt, 60-cycle alternating current passed through the tissue between the 2 platinum hooks. Stimulation was applied for a 5-second period once every 2 to 4 minutes, depending upon the speed of a single contraction-relaxation cycle, which also varies with temperature. Since a stimulation-potential of 10 to 15 volts causes maximal amplitude of contraction, the 20-volt stimulus is supermaximal, and thus it may be assumed that the whole mass of contractile substance is reacting. The relatively high potential also assures a pattern similar to that of spontaneous contractions. After recording 3 contractions at a given temperature, the temperature of the water bath surrounding the tubes was changed in about 2 minutes and an additional 8-minute period was allowed for adjustment to the new temperature. Observations were begun at 35°C and the temperature was decreased by 5°C at each step to 5°C. Then, in order to exclude changes in amplitude due to fatigue the temperature was raised by 5°C steps to 45°C, recording 3 contractions at each temperature as before.

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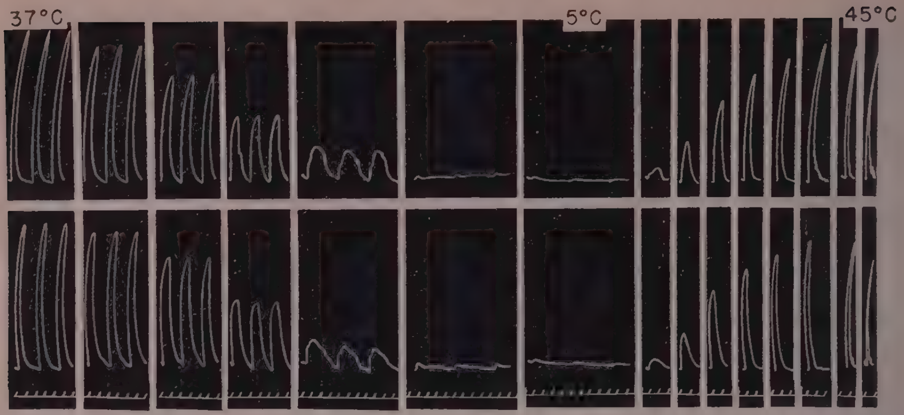

FIG. 1. Amplitude of electrically-stimulated contractions. Simultaneous runs with identical strips from an estrous rabbit. Temperature changes, by 5° steps, indicated by vertical white spaces. Time intervals, 1 min.

After several preliminary experiments on sows' and rabbits' uteri, 8 strips were recorded from 3 rabbits in estrus and from one 28 days pregnant. Fig. 1 represents two runs. As the temperature is decreased the amplitude (extent of shortening) simultaneously decreases step by step until at 5°C the shortening, even though magnified by the lever, is scarcely noticeable. As the temperature is again increased, the amplitude increases up to 35°C. At 40°C however, the behavior of the muscle changes and instead of increase in amplitude there is a decrease, which goes further with increasing temperature. It can also be seen that the speed of both contraction and relaxation decreases with decreasing temperature.

The experimental apparatus and conditions having been carefully standardized, it is possible to combine the results in a plot of amplitude against temperature. Fig. 2, summarizing the outcome of 14 experiments on the 8 strips used, shows graphically the relation described above. This curve has two directions of slope from the peak at or near 35°C. Only that phase between 5° and 35° has thus far been studied in detail.

Fig. 3 compares the amplitude of spontaneous contractions with those electrically induced and shows they are alike both in amplitude and in the speed of contraction and relaxation. This indicates that the reaction (or series of reactions) resulting in spontaneous contraction is also dependent upon temperature.

The amplitudes in the temperature range 10°-35°C were plotted against 1/T, by the formula log A/B in which A is the recorded amplitude at each temperature and B the maximal amplitude minus A. A straight line was obtained as in the work on skeletal muscle cited above. It should, however, be mentioned that use of the logarithmic quantity tends to lessen the fluctuations of the curve and thus to straighten it out.

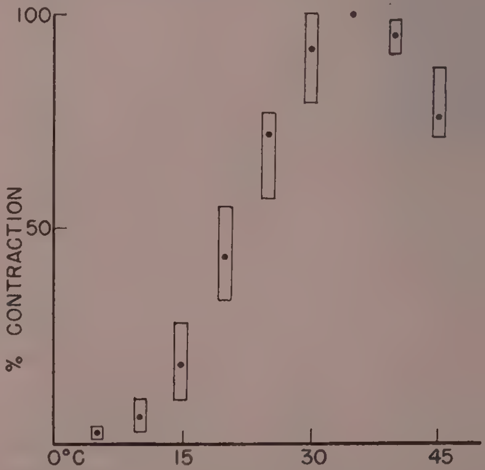

FIG. 2. Extent of contraction at each temperature, plotted as % of maximum, against the temperature. Dots indicate averages of 14 experiments, bars indicate ranges observed.

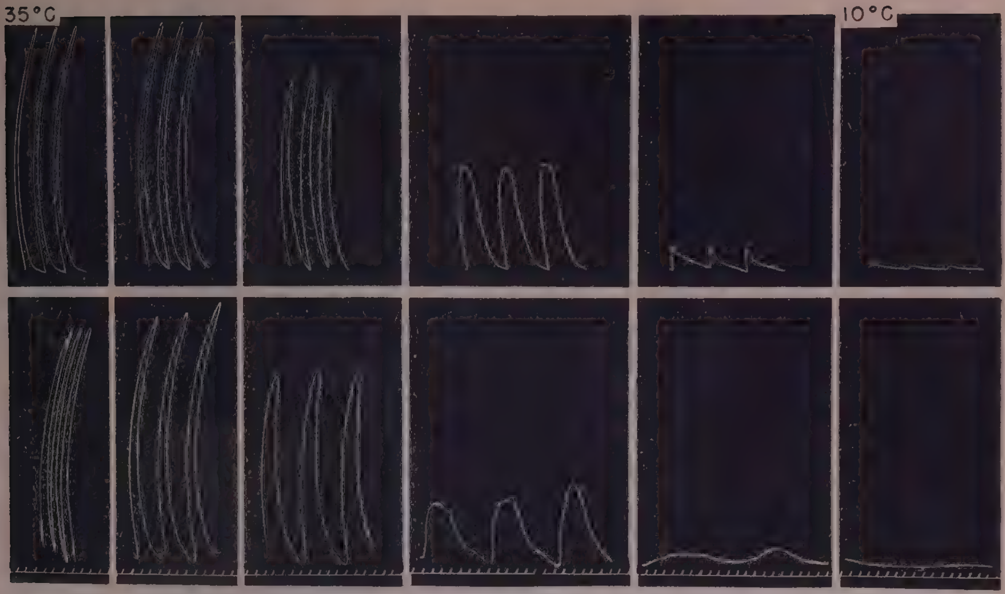

FIG. 3. Comparison of spontaneous with electrically-stimulated contractions. Rabbit 28 days pregnant. Temperature changes, by 5° steps, indicated by vertical white spaces. Time intervals, 1 min.

Summary. In agreement with the observations of others on skeletal muscle, strips of uterine muscle from rabbits in estrus undergo changes in the extent of shortening in relation to temperature. The speed of contraction and relaxation also varies with temperature. These effects occur equally whether the contractions are spontaneous or electrically simulated.

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Invasiveness of Fibrosarcomata in Mice.* (19042)

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It has been shown that the characteristic of invasiveness of chemically induced fibrosarcomas (the presence of discrete nodules in the abdomen derived either by direct extension of the tumor or by the process of metastasis) is partially determined by some new principle found in the host which had been derived from its ancestry and differentially determined by litter seriation(1). These conclusions were based upon observations on 788 mice of 2 descents (Prunt and 2Prunt), which were genetically related to each other (17 generations of inbreeding in common).

In the first analysis of an association between invasiveness of fibrosarcomas and litter

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TABLE I. Summary of Development of Invasive Tumors in Mice Showing Stock, Litter, Total No. of Mice Showing Invasive Tumors (I); Total No. of Fibrosarcomas (T); and % of Mice Which Developed Invasive Tumors (%). Data of 1-7, 8-15 and 1-15 Are Also Combined.

	Prunt			2 prunt			Total		
Litter	T	I	%	T	I	%	T	I	%
1	80	8	10	86	8	9.3	166	16	9.6
2	114	9	7.9	105	11	10.5	219	20	9.1
3	130	9	6.9	171	11	6.4	301	20	6.6
4	146	11	7.5	172	16	9.3	318	27	8.5
5	125	3	2.4	157	14	8.9	282	17	6
6	69	7	10.1	113	7	6.2	182	14	7.7
7	43	1	2.3	99	7	7.1	142	8	5.6
8	17	0		56	3	5.4	73	3	4.1
9	9	0		29	1	3.4	38	1	2.6
10	10	0		7	0		17	0	0
11	5	0		4	0		9	0	0
12	1	0		2	0		3	0	0
13				6	0		6	0	0
14				4	0		4	0	0
15				2	0		2	0	0
1–7	707	48	6.79	903	74	8.19	1610	122	7.6
8–15	42	0		110	4	3.63	152	4	2.6
1–15	749	48	6.41	1013	78	7.70	1762	126	7.15

seriation (mother's age ?), the data on some of the litters were small and it became necessary to add successive litters 1 + 2, 3 + 4, 5 + 6, 7 + 8, 9 + 10 and 11 + 12, together in order to show smoother trends. There were also slight differences between the sexes and between the 2 descents, which, however, were not statistically significant.

In the present analysis dealing with the same phenomenon, 974 new data were added. The combined data on 1,762 mice are given in Table I, according to litter of origin.

The origin and genetic relationship of the Prunt and 2Prunt descents have already been described(1). All mice were injected with 1 mg of methylcholanthrene dissolved in 0.1 cc of sesame oil at 60 days of age and continued as breeders. The young, however, were discarded shortly after birth. The mice were examined weekly for tumors beginning 2 months following the injection of the carcinogen. The latent period was determined by the appearance of a solid firmly attached progressively growing nodule at the site of the injection. The mice were continued until death at which time the tumor was measured in three dimensions, a complete autopsy performed and all pathological tissues saved for histological examination. Only mice bearing fibrosarcomas are included in this analysis, which is based upon the local growing tumor at the site of the injection of the carcinogen with or without the presence of secondary nodules of similar histological appearance in the abdomen.

The percentage of mice developing fibrosarcomas which have the capacity of invading into the abdomen continue to show the same down-trend with successive litters as was found in the original series. There is no significant difference between the 2 descents. There were 6.41% invasive tumors in the Prunt descent compared to 7.70% for mice of the 2Prunt descent. The percentage of invasive tumors for mice of the sixth litter of

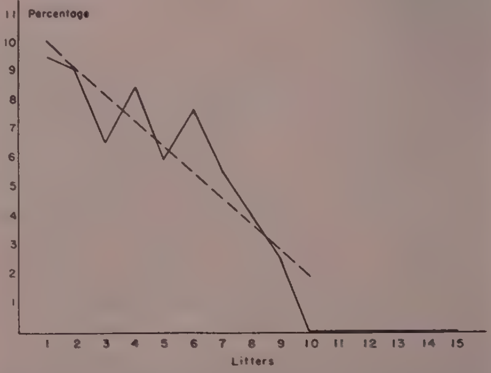

FIG. 1. % of mice showing invasive fibrosarcomas following subcutaneous injection of methylcholanthrene. Actual data are plotted on solid line; fitted straight line (dash) has a slope value of 5.5.

the Prunt descent seems to be out of trend. Nevertheless it seems permissible to combine the 2 series. The data of Table I are plotted in Fig. 1 and the slope of the combined data is also given (slope 5.5). This slope has a statistically significant difference from slope of "O".

Data for litters 1-7 were combined and compared to similar data for mice of the 8-15th litters. This comparison shows a "t" value of 2.3 which is statistically significant at the 5% level.

The new data thus permit a verification of the correlation reached in the earlier series. There appears to be a gradual loss of ability of a mouse to develop an invasive fibrosarcoma in the successive litters born to the same parents, when subjected to 1 mg of methylcholanthrene dissolved in 0.1 cc of sesame oil at 60 days of age. There is a complete loss of this ability in mice of the 8th and later litters of the Prunt descent, and in mice of the 10th and later litters of the 2Prunt stock. The present phenomenon of changes in invasiveness may also be interpreted by mice of successive litters acquiring an increased resistance to chemically induced fibrosarcomas.

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Action of Coumingine on Heart Muscle.* (19043)

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It is often stated that the erythrophleum alkaloids are the only alkaloids that have a digitalis-like action. Chemically these alkaloids have the cyclopentenophenanthrene ring system common to the digitalis glycosides, but do not contain the unsaturated lactone group said to be responsible for digitalis action. However, the usually accepted criteria of digitalis action, slowing of the heart, systolic arrest and emesis following parenteral administration, cannot be considered adequate, none being specific. Straub(1) long ago emphasized that "so-called systolic standstill" was not a specific digitalis action. More recently Pisanty(2) has studied the systolic arrest and skeletal muscle shortening caused by a variety of substances and the enzyme mechanisms involved. Observations to date(3,4) have indicated that the constant, and perhaps specific, measurable action of digitalis glycosides in experimental preparations and in therapeutic usage in man is to shorten the refractory period, or systole, of heart muscle without impairment of contractility. In their study of the erythrophleum alkaloids, Maling and Krayer(5) found the outstanding action was to decrease diastolic volume. Coumingine had a positive inotropic effect in lower concentrations, but a greater tendency to cause irregularity of rhythm than the other alkaloids of the group. They concluded that as yet there was no evidence to justify the assumption that in congestive heart failure the action of erythrophleum alkaloids was truly digitalis-like.

The present paper reports observations on the effect of coumingine on the primary properties of heart muscle of the turtle *Pseudemys*

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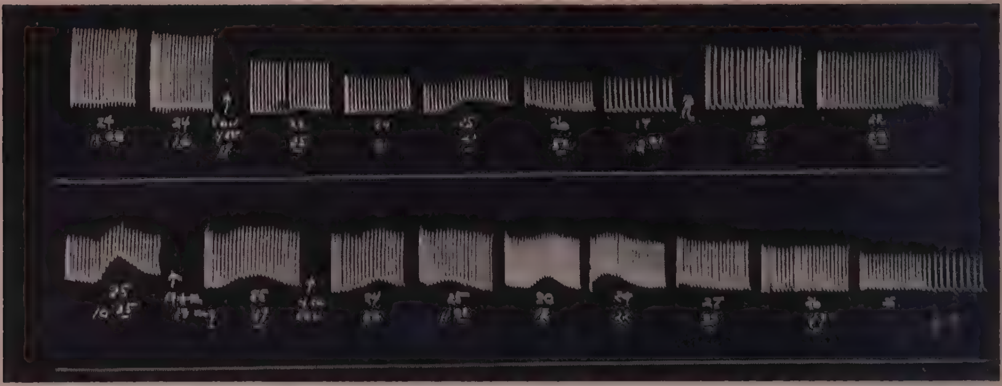

FIG. 1. A. Upper line: The response of a spontaneously beating auricle to coumingine 1:.6 million, at arrow. At R bath was changed to fresh Ringer solution. B. Lower line: The response of an atropinized auricle to the same concentration of coumingine, at second arrow. Rate increased 25 min after addition of drug. Upper figures, rate; lower, time.

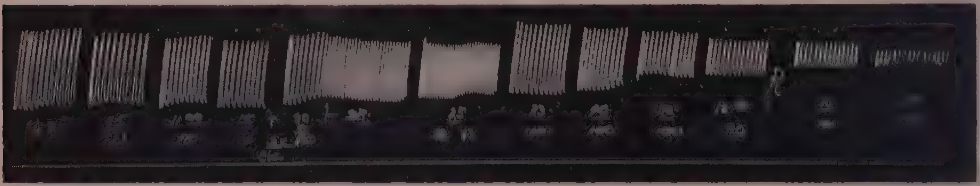

FIG. 2. The response of a rhythmically driven ventricular strip to coumingine 1:1 million, at arrow. Driven rate, 14 per min. SR, spontaneous rhythm, rate 33. Upper figures, time; lower, relative threshold readings.

elegans. With none of the drugs studied to date in this laboratory has there been the variability of response and want of relation between drug concentration and intensity of action that has been exhibited by coumingine. The drug has a pronounced vagal action on the auricle. Shortening of ventricular muscle was its outstanding action. While this effect might be expected to increase cardiac output in the dilated heart, the tendency to develop tachycardias would preclude the use of the drug in clinical medicine. The characteristic shortening of refractory period produced by digitalis bodies was not seen.

Action on auricle. The response of the isolated spontaneously beating auricle to concentrations ranging from 1:20 to 1:0.5 million was very variable. The most consistent action was a vagal negative inotropic effect. Vagal slowing or stoppage occurred with high concentrations, either early or 45 minutes to an hour after the drug had been added. Recovery followed atropine or at times merely changing to fresh Ringer solution (Fig. 1A). In one experiment stoppage was followed by tachycardia. Occasionally there was a positive inotropic action in the stimulated auricle; that action appeared late and then soon declined. Diastolic shortening was not marked, even with high concentrations; it was more apt to appear in the atropinized auricle. Tachycardias occasionally appeared with high concentrations of the drug, again more frequently after atropine (Fig. 1B).

Action on ventricle. With concentrations up to 1:10 million spontaneous tachycardias and extrasystoles often appeared in driven ventricular strips, usually soon after the addition of the drug (Fig. 2).

The effect on contractility was studied in rhythmically stimulated ventricular strips suspended in a bath and the beat recorded by a kymograph. Diastolic shortening, with concentrations as low as 1:40 million, was the constant irreversible action of the drug. Transient slight positive inotropic effects were oc-

TABLE I. Effect of Coumingine on Beat Size and Threshold for Electrical Stimulation.

Exp.	Concentration, parts/million	Min after drug	% decrease in beat size: Systolic	% decrease in beat size: Diastolic	% of basal threshold
1	1: .8	50	52	34	354
2	1: .9	50	11	22	180
3	1: 1	45	0	58	82
4	1: 2	45	25	50	128
5	1: 2	30	29	43	160
6	1: 5	40	35	28	140
7	1: 5*	50	26	20	140
8	1:10	60	0	52	200
9	1:10*	60	0	22	150
10	1:20	60	12	54	75
11	1:40	75	12	56	58

* Glutathione added to bath before coumingine.

casionally seen, and when tachycardias appeared early, beat size was well maintained with the increased rate. Even late, systolic peaks usually were not greatly lowered, reduced beat size being largely due to diastolic shortening (Table I). When such muscle shortening is due to action on SH groups Pisanty(2) found that it could be prevented by certain sulfhydryl compounds. The shortening caused by coumingine was not prevented by the previous addition to the bath of glutathione or cysteine in relatively high concentrations, *e.g.* the presence of glutathione 1:3000, did not prevent shortening and rise in threshold by coumingine 1:10 million. In general, the tolerance of auricular muscle to this alkaloid greatly exceeded that of the ventricle. The difference did not appear to be related to rate of beating or to the effect of stimulation.

The effect on the threshold for electrical stimulation was also studied in those experiments in which contraction was recorded. With this type of preparation a basal threshold is usually reached about one-half to an hour after beginning stimulation. Control experiments indicate an expected rise to 120% of the basal threshold after an additional 2 hours of stimulation. In most experiments with higher concentrations coumingine caused a definite rise (Table I). The raised threshold is probably concomitant with depression of conductivity (Table II).

The effects on latent period, refractory period and conduction were studied in rhythmically driven strips from which two unipolar electrograms were simultaneously recorded, using small wick electrodes with an inter-electrode distance of about 25 mm. The Q-T interval of such electrograms has been shown to be a reliable measure of the refractory period of that area of muscle. Normally, there is shortening of about 8% per hour after the first hour due to driving itself. All noxious agents appear to shorten refractory period. The results of experiments given in Table II show only a single example in which there was shortening of refractory period comparable to that produced by digitalis glycosides. The effect on conduction was followed by changes in the Q-Q interval of these electrograms. An appreciable rise in conduction time was the rule (Table II).

Summary. The action of the erythrophleum alkaloid coumingine has been studied on the turtle heart. It has a pronounced vagal effect on the auricle. Diastolic shorten-

TABLE II. Effect of Coumingine on Latent Period, Refractory Period and Conduction Time.

Exp.	Concentration, parts/million	Min after drug	% change of basal intervals: S–Q	% change of basal intervals: Q–T	% change of basal intervals: Q–Q
1	1: .4	70	+58	— 6	— 14
2	1: .5	50	NC	NC	+ 41
3	1:1	35	—	+ 3	—
4	1:1	60	—40	—11	+ 66
5	1:1	40	+16	—33	+300
6	1:1.5	50	NC	—14	+112
7	1:2	60	+33	+ 7	+ 33
		90	+66	+ 3	+100
8	1:10*	40	—	— 4	+ 22
9	1:10*	60	—	+ 5	+ 12

S–Q, latent period, shock to Q interval. Q–T interval, refractory period. Q–Q interval, conduction time for inter-electrode distance.

* Spontaneous rhythms appeared during experiments.

NC = no change.

ing of ventricular muscle was the outstanding action. Conduction time in ventricular muscle was prolonged and the threshold for electrical stimulation raised. The characteristic shortening of refractory period produced by digitalis glycosides did not occur. The tendency to cause spontaneous tachycardias would preclude the use of coumingine in clinical medicine.

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Failure of Transfusion of Hemophilic Plasma to Induce Abnormalities of Hemostatic Mechanism in Normal Individuals. (19044)

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Most investigators agree that the fundamental defect in hemophilia is represented by the deficiency of a specific plasmatic factor(1). This factor has been variously defined as "antihemophilic globulin", "thrombocytolysin", "plasmakinin", "thromboplastinogen", etc. Much experimental evidence has accumulated in support of this theory, the most significant observation being perhaps that small amounts of "antihemophilic globulin" are able to normalize temporarily the clotting defect of hemophiliacs. On the other hand, some evidence has been presented to show that hemophilia might be due to the presence of a circulating anticoagulant; a significant finding in support of this theory is that hemophilic plasma may be made to clot normally by simple dilution with physiological saline(2).

The favorable effect of transfusion of fresh normal plasma on the clotting activity of hemophiliacs is well known. On the other hand, the effect of transfusion of hemophilic plasma on the clotting mechanism of normal subjects does not seem to have been investigated. Should a circulating anticoagulant be responsible for the clotting defect of hemophilia, the transfusion of sufficient amount of hemophilic plasma in normal subjects could be expected to be followed by detectable abnormalities of the coagulation mechanism.

Materials and methods. Two patients with classical hemophilia as confirmed by clinical and laboratory data were used as donors in this study. Both patients presented the usual response to transfusion of fresh human plasma and did not show precipitating antibodies(3) against human plasma or "antihemophilic globulin". Five hundred ml of blood were collected in Baxter vacuum bottles containing 100 ml of ACD solution, the donors fasting for at least 4 hours prior to donation. The blood was centrifuged at 2,000 r.p.m. for one hour in refrigerated centrifuge and the plasma separated with sterile technic. Within 2 hours from the time of collection the plasma was injected into compatible normal subjects. An amount of 5 ml of plasma per kilo weight was administered at a speed of 150-200 drops per minute. In the hemophiliacs prior to donation and in the normal subjects before, immediately after the transfusion and one hour later the following determinations were made: (a) bleeding time, according to the method of Ivy *et al.*(4); (b) tourniquet test, according to the method of Frugoni and Giugni(5), maintaining a pressure equal to the mean pressure of the subject for 10 minutes; (c) platelet count, according to the method of Dameshek(6); (d) clotting time of whole blood in glass and silicone-coated test tubes, according to a modification of the method of Lee and White (7); (e) prothrombin activity of plasma and serum, according to the procedures recently described by one of us(8,9); (f) activity of plasma prothrombin conversion factor (PPCF, labile factor) in plasma and serum, according to the methods of one of us (8,10); (g) accelerator effect of serum, by the method of de Vries *et al.*(11); (h) plasma fibrinogen level, by a slight modification of the method of Quick(12); and of the fibrinolytic activity of plasma, by the method of one of us(13); (i) ability of plasma collected from normal subjects after the transfusion with hemophilic plasma to correct the coagulation defect of known hemophiliac *in vitro*. Citrated (0.02 M) plasma under study and citrated plasma of known hemophiliac were mixed in various proportions to a total volume of 0.9 ml; 0.1 ml of $CaCl_2$ M were added and the clotting time of the mixture and the prothrombin activity of the serum one hour after the completion of coagulation determined; (j) effect of the addition of plasma collected from normal subjects after the transfusion with hemophilic plasma on the coagulability of normal plasma *in vitro*. Citrated plasma from normal individual was mixed with citrated plasma obtained from normal subjects after transfusion of hemophilic plasma, in various proportions to a volume of 0.9 ml. The rest of the procedure was as described under paragraph (i); (k) changes in the electrophoretic pattern of plasma from normal subjects after transfusion with hemophilic plasma. Hemophilic plasma presents a characteristic pattern(14), which was found in plasma from both hemophiliacs used in this study. The technic employed was that of Bernfeld *et al.*(15).

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* Damon Runyon Senior Clinical Research Fellow.

† Fellow of the Rockefeller Foundation.

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Results. The results obtained in these experiments are summarized in Tables I, II and III, where the findings in one case are reported. The other case gave essentially similar data. Both normal subjects received an amount of hemophilic plasma equal to approximately ⅛ of their calculated plasma volume. No abnormality of the hemostatic mechanism was detected in normal recipients following the transfusion. No bleeding manifestations of any kind were noted; bleeding time, capillary fragility and the coagulation factors examined were found unchanged after the transfusion. No alterations of the normal electrophoretic pattern were noticed. Moreover, it is significant that plasma collected from normal subjects immediately after the transfusion of hemophilic plasma was still able to correct temporarily the coagulation defect of a known hemophiliac. It also did not retard in any way the coagulation process of other normal plasma *in vitro*. To exclude the possibility of any delayed effect, one recipient was studied 24 hours after the transfusion of hemophilic plasma. Again, no abnormality of the hemostatic mechanism could be detected.

A drop in the platelet count was noticed immediately following the administration of plasma; the platelet count gradually rose and came back to normal level in 3 hours. Such a phenomenon cannot, however, be specifically attributed to the transfusion of hemophilic plasma. It has been observed by us that a temporary drop in the platelet level occurs in the majority of normal subjects receiving normal plasma(16).

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TABLE I. Effects of the Transfusion of Fresh Hemophilic Plasma in Normal Subjects.

	Hemophiliac	Normal		
		Before transfusion	After transfusion	1 hr later
Red blood cells, millions/cmm	2.95	4.01	3.86	3.79
White blood cells/cmm	8700	7100	7500	9000
Polymorphs, %	65	60	58	61
Lymphocytes, %	29	27	32	34
Monocytes, %	5	9	8	5
Eosinophiles, %	1	2	1	1
Basophiles, %	0	2	1	0
Platelets/cmm (× 10,000)	64.8	44.1	27.02	35.24
Hematocrit, %	30	39.5	38	37
Bleeding time, min	8	4½	5	3½
Tourniquet test, petechiae*	12	6	8	5
Clotting time of whole blood glass test tubes, min	59	8	7½	7
Silicone-coated test tubes, min	470	25	18	22
Prothrombin activity, plasma, %	95	100	100	100
" " serum, %	90	2.4	2	3.5
PPCF, plasma, %	80	95	100	90
" serum, %	65	4.5	12	0
Accelerator effect of serum, %	14.5	75.6	92.6	70.8
Plasma fibrinogen level, mg %	372.8	342.6	297.5	354.9
Plasma fibrinolytic activity, %	4	8	17	9.5

* No. of petechiae on volar surface of forearm in area 1 inch diam., after application of mean arterial pressure for 10 min.

TABLE II. Ability of Plasma Collected from Normal Individual Before and After Transfusion of Hemophilic Plasma to Correct Coagulation Defect of Known Hemophilic Plasma.

(a)	Hemophilic plasma, vol	10	9	5	1	0
	Plasma from normal individual, vol	0	1	5	9	10
	Clotting time of recalcified plasma, min	22	5	3½	3½	3
	Prothrombin activity of serum, %	90	72.5	33.7	18.2	3
(b)	Hemophilic plasma, vol	10	9	5	1	0
	Plasma from normal individual, inj. with hemophilic plasma, vol	0	1	5	9	10
	Clotting time of recalcified plasma, min	23½	4½	3½	2½	2½
	Prothrombin activity of serum, %	87.5	68.4	40.2	12.5	4

TABLE III. Clotting Ability of a Normal Plasma *in vitro* After Addition of Various Volumes of Plasma Collected from a Normal Individual Receiving a Transfusion of Hemophilic Plasma.

(a)	Normal plasma, vol	10	9	5	1	0
	Plasma from normal individual, before transfusion of hemophilic plasma, vol	0	1	5	9	10
	Clotting time of recalcified plasma, min	2¼	2½	2½	2½	3
	Prothrombin activity of serum, %	.9	2.3	2	1.8	3
(b)	Normal plasma, vol	10	9	5	1	0
	Plasma from normal individual, after inj. of hemophilic plasma, vol	0	1	5	9	10
	Clotting time of recalcified plasma, min	3	2¼	2½	2½	2½
	Prothrombin activity of serum, %	2.1	1.3	.9	2	1

It is realized that in experiments of this kind a circulating anticoagulant may fail to reveal its presence through excessive dilution. With this possible limitation, our findings fail to support the view that an inhibitory or anticoagulant factor represents the fundamental defect in hemophilia.

Summary. In an attempt to demonstrate the presence of a circulating anticoagulant in hemophilia, normal human subjects were

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16. Stefanini, M. and Chatterjea, J. B., to be published.

transfused with compatible hemophilic plasma. No effect on the hemostatic mechanism of normal subjects was demonstrated following the transfusion, nor did the ability of the recipient's plasma to correct the clotting defect of known hemophilic plasma change following this procedure.

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Anticonvulsant Activity of 2,2-Disubstituted-1,3-Propanediols in Electroshock Seizures. (19045)

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It has been recently shown that certain 2,2-disubstituted-1,3-propanediols possess marked anticonvulsant properties against convulsions produced by pentylenetetrazole (metrazol) (1,2). One compound of this series, 2,2-diethyl-1,3-propanediol (DEP) which received a more thorough pharmacological investigation(1-4) proved very effective against convulsions produced by chemical agents such as strychnine, pentylenetetrazole and picrotoxin, but was relatively weak in preventing electroshock seizures(1,4). It was of interest to investigate whether all 2,2-disubstituted-1,3-propanediols would show stronger anticonvulsant activity in chemoshock convulsions than in electroshock convulsions or whether this property was peculiar to DEP. In view of the close chemical relationship of certain 2,2-disubstituted-1,3-propanediols to barbiturates, the possibility that each of these compounds may be converted in the body to the same anticonvulsant substance was considered.

Experimental. White male mice of the CF-1 strain weighing between 18-24 g were used. Seizures were produced by applying through corneal electrodes 60 cycle current from a Hans-Tech electroshock apparatus with a current intensity of 50 milliamperes and 0.2 second duration. With the machine used the current delivered was independent of the external resistance. Experiments have shown that with a stimulus of 12.5 milliamperes and duration of 0.2 second about 50% of mice convulse; thus 50 milliamperes represents about 4 times the convulsive threshold dose. Most mice survived repeated shocks of 50 milliamperes administered at hourly intervals without showing marked changes in sensitivity to electroshock seizures. Drugs were administered orally in a 5% suspension of gum acacia. Groups of 10 mice were treated at dose levels increasing in geometrical progression by a factor of approximately 1.5 and shocked 30, 90 and 150 minutes after the administration of the drug. The number of animals protected at each dose level was noted. A mouse was considered to convulse if it showed the extensor tonic phase of the convulsion as described by Toman *et al.*(5). Mice showing only clonic seizures are considered non-reactors. The results were obtained graphically(6) and are expressed as the dose protecting 50% of the animals from the tonic extensor phase of electroshock convulsions. The compounds used in these studies were prepared by Dr. B. J. Ludwig and his associates of these Laboratories.

Results. The table gives the doses in mM kg protecting 50% of animals from the extensor tonic phase of electroshock seizures, applied 30 minutes after administration of

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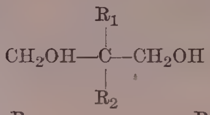
TABLE I. Mean Protective Doses of Substituted Propanediols in Electroshock Seizures and Pentylenetetrazol Convulsions in Mice.



$$CH_2OH-\overset{R_1}{\underset{R_2}{C}}-CH_2OH$$

No.	R_1	R_2	Molecular wt	Electroshock, protective dose, oral, mM/kg	Metrazol, protective dose, intraperitoneal, mM/kg
1	methyl	methyl	104	>20.6	>2.5
2	ethyl	ethyl	132	2.8 ± .08	.3
3	*n*-propyl	*n*-propyl	160	2.6 ± .17	.55
4	*n*-butyl	*n*-butyl	188	2.9 ± .19	>1.3
5	methyl	ethyl	118	5.8 ± .41	1.35
6	”	*n*-propyl	132	2 ± .1	.53
7	”	iso-propyl	132	3.2 ± .28	.67
8	H	*n*-butyl	132	5.8 ± .3	—
9	methyl	”	146	3.2 ± .21	1.44
10	ethyl	”	160	3.4 ± .23	—
11	”	ethoxy	148	8.7 ± .22	>1.7
12	phenyl	H	152	3.2 ± .18	>1.6
13	”	ethyl	180	1.2 ± .04	.67
14	phenoxy	”	196	4.5 ± .31	>1.3
15	phenyl	phenyl	228	1 ± .04	—
16	benzyl	benzyl	256	3.1 ± .3	—
17	phenobarbital sod.		254	.16 ± .008	.15
18	mephenesin		182	2.3 ± .12	.44

the compound. With the exception of phenobarbital, all compounds were ineffective 90 and 150 minutes after administration. The doses protecting animals from death caused by pentylenetetrazole 100 mg/kg as previously found(1) are also given.

Compounds with aliphatic substituents on the C_2 atom did not show outstanding anticonvulsant activity. The dimethyl substituted compound was inactive and the methyl ethyl compound had only slight activity. Compounds with 2 aliphatic substituent groups having a total of 4 or more carbons showed anticonvulsant activity of a comparable degree; it appeared to be of little importance whether the substituent groups were placed symmetrically or asymmetrically around the central carbon atom. Anticonvulsant action was decreased when one of the substituents was H or an alkoxy group as in compounds No. 8 and 11. It was of interest to note that DEP (No. 2) which on a weight basis possessed a similar protective action as phenobarbital against convulsions produced by pentylenetetrazole, was about 10 times less effective than phenobarbital when tested by the electroshock procedure. The methyl propyl isomer (No. 6), which was less effective in chemoshock appeared significantly more active in the electroshock procedure. The methyl isopropyl isomer (No. 7) was of somewhat lower efficacy than DEP in both electroshock and chemoshock tests. The *n*-butyl isomer (No. 8) had only weak anticonvulsant action.

Among the compounds with aromatic substituents the phenyl ethyl and the diphenyl propanediol possessed an anticonvulsant activity almost 3 times that of DEP in preventing electroshock seizures. Compounds where one of the substituents was an H or aryloxy group (No. 12 and 14) showed less activity. The dibenzyl propanediol had also a relatively weak anticonvulsant action.

Discussion. A study of Table I indicates that all compounds possessing anticonvulsant activity of some magnitude possess in common a carbon atom to which four other carbon atoms are directly attached (a quaternary carbon atom). This is a common feature of anticonvulsant drugs of both the barbiturate and propanediol series. Compounds with a tertiary carbon atom such as No. 8, 11, 12 and 14 are less anticonvulsive than comparable compounds possessing a quaternary carbon. Thus 2-*n*-butyl-1,3-propanediol (No. 8) is considerably less potent as an anticonvulsant than the 3 isomers possessing quaternary carbon atoms. Similarly the phenyl ethyl propanediol is more effective than the phenoxy ethyl analogue. However, propanediols possessing a quaternary carbon are not necessarily anticonvulsant as is witnessed by the lack of activity of the 2,2-dimethyl-1,3-propanediol (No. 1).

Putnam and Merritt(7) have stated that practically all substances with marked anticonvulsant properties contain at least one phenyl nucleus in a terminal position. If two phenyl nuclei are present they are both attached to the same carbon atom. It is of interest that in conformity with this rule the propanediols containing one or two phenyl nuclei show strongest anticonvulsant activity.

The propanediols differ from other anticonvulsants of the barbiturate, hydantoin, and oxazolidine-2,4-dione series in possessing a very short duration of action. When animals were repeatedly subjected to electroshock seizures, propanediols were effective 30 minutes after administration of the drug but gave no protection 90 or 150 minutes after administration. Mephenesin showed a similarly short duration of action. Phenobarbital on the other hand had a long duration of action and full activity came on slowly.

It is of interest to speculate whether compounds related to substituted 1,3-propanediols occur as degradation products of certain barbiturates in the body. It may be that for instance phenobarbital is broken down in the body to urea and a product related to 2-ethyl-2-phenyl-1,3-propanediol. Goodman and Toman(8) have pointed out that no unequivocal proof has ever been advanced to indicate that antiepileptic drugs act *per se* to prevent seizures and it is within the realm of possibility that a compound derived from 2-ethyl-2-phenyl-1,3-propanediol is the effective anticonvulsant agent of phenobarbital. The effectiveness of the barbiturates and the propanediols in both electroshock and chemoshock convulsions certainly appears to point in this direction.

Summary. 2,2-Disubstituted-1,3-propanediols in suitable dosage prevent electroshock convulsive seizures in mice. The molecular configuration favorable for maximal anticonvulsant activity is the presence of a quaternary carbon atom to which one or two phenyl radicals are attached. The possibility is pointed out that derivatives of propanediols occur in the body as degradation products of certain barbiturates and they may constitute the effective anticonvulsant substances.

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Effect of Intratracheally Administered Wulfenite on Lungs of Mice. (19046)

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Evans(1-3) reported that the introduction of piezo-electrically active materials into the tracheae and peritoneal cavities of rats and of rabbits resulted in extensive proliferation of fibrous tissue in the lungs and in the abdomen. This reaction was thought to result from the electromotive charges given off by the dusts when exposed to stresses and strains in the body tissues. Evans felt that this property might explain the pathogenesis of pneumoconiosis, since Lanza's data(4) indicated that

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only dusts possessing the property of piezoelectricity are capable of inducing pneumoconiosis. This hypothesis has been adopted by Chess(5) as a possible explanation for the pathogenesis of regional enteritis. Since the original work has not been verified the present experiment was performed.

Methods. The piezoelectrically active material wulfenite was obtained from the National Bureau of Standards and processed in the fashion described by Evans. The material was ground by hand until 35% passed through a 200-mesh screen and it was then suspended in distilled water. Thirty-eight littermate mice of the Sprague-Dawley strain, approximately three months old, were then anesthetized with ether and 0.1 cc of the suspension (25 mg of wulfenite) was injected directly into the trachea of each animal through an 18 gauge needle. Four animals were then sacrificed at 2 to 4-week intervals over a period of 5 months. The lungs were removed and examined histologically.

Results. At autopsy the lungs of 34 of the mice appeared normal. During the later months 4 of the animals showed areas of pneumonia and an occasional emphysematous bleb. Microscopic examination of the lungs of all of the animals revealed small particles of wulfenite scattered at random, and in high concentration, throughout the lungs of all but 4 of the mice. In the latter no particles were seen. In no instance was there any suggestion of fibrous tissue reaction to this accumulated material. nor was there evidence of inflammatory reaction. The photomicrograph (Fig. 1) is typical of the lungs containing wulfenite.

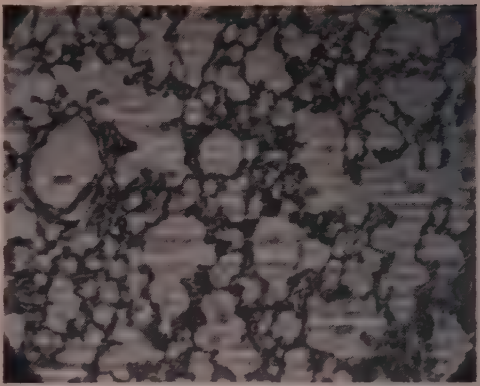

FIG. 1. Mouse lung containing particles of wulfenite scattered through normal alveoli without evidence of fibrous tissue reaction.

Discussion. Under the conditions of the above experiment wulfenite failed to elicit any fibrous tissue reaction in the lung. Although the maximum particle size (0.074 mm) was larger than that reported by Evans (.049 mm) many particles of very small size were evident in the sections. In no instance was there any form of cellular reaction to these particles. A species difference in susceptibility to piezoelectrically active materials could possibly account for the discrepancy in the results since mice were used in this experiment, whereas Evans used rats and rabbits.

Summary. The piezoelectrically active dust, wulfenite, of maximum particle size .074 mm was administered intratracheally to 38 mice. In no instance was there evidence of cellular reaction to the wulfenite in the lungs of these animals. The theory that a substance, by virtue of its possession of piezoelectric properties, can produce reaction in the living organism requires further confirmation.

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Presence in Tumor Tissue of a Mouse Mammary Cancer Accelerant.* (19047)

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In studies of the effect of tissue extracts on transplantable tumors, it is known that the preparation of the recipient host by the injection of extracts may result in an acceleration of the growth of homologous tumor tissue upon its transplantation into susceptible hosts (1-7), or that it may cause normally resistant hosts to become susceptible to a homoiotransplant tumor(13-15,17,20,21). These extracts were derived from frozen(1-7,13,17) or lyophilized tumor tissues(14,15,20,21), or even from normal tissues(14,15). Casey (1-9) designated the observed enhancement, or accelerative effect, the "XYZ phenomenon", and the factors derived from frozen tissue which elicit enhancement as the "XYZ factor." It is significant to the present report that the attempts to date to demonstrate conclusively enhancive, or accelerative, factors in fresh unfrozen tumor extracts as potent as those derived from frozen tumor tissue have been uniformly unsuccessful(8-11,16,18,19).

The present work was undertaken with the purpose of exploring by physical, chemical, and immunological methods the factors responsible for enhancement and for the retardation in growth of tumor cells. This report makes it known that accelerant factors for a transplantable mouse mammary carcinoma of inbred C_3H mice are equally effective whether derived from fresh (unfrozen) or frozen homologous cancerous tissues.

Materials and methods. Tumor. The accelerative test materials and the transplantable tumor tissue employed for challenge inoculation were derived from a mouse mammary adenocarcinoma, Z8352. This tumor had arisen spontaneously in a C_3H female possessing the milk agent. At the time this report was written, it had been maintained for 13 successive passages by transplantation in susceptible ZBC mice[‡] known not to possess the milk agent. *Hosts.* The mice employed as test animals were from ZBC stock, assumed, in the absence of direct testing, to be free from the milk agent. This stock supports regularly the growth of Z8352 tumor cells on transplantation. *Preparation of accelerant factors.* The accelerant factors from fresh (unfrozen) tumor tissue were prepared as follows: A freshly excised Z8352 tumor was passed through a tissue press to yield a mince. This mince was triturated by means of a mortar and pestle, using a minimum of sterile alundum as an abrasive, and suspended in 0.85% saline solution in the ratio of 3 ml of saline per g of tissue mince. The suspension was homogenized by means of a Potter-Elvehjem homogenizer and then centrifuged horizontally at 1200 r.p.m. for 15 minutes. The supernatant fluid was subjected to four successive centrifugations, the sedimented material being discarded after each run. Following the fourth and final centrifugation, the supernatant fluid was employed as a source of "fresh accelerant." The method employed for the preparation of accelerant factors from frozen tumor tissue was essentially similar to that outlined by Casey in his preparation of the XYZ factor (1-7). Freshly excised Z8352 tumor tissue was placed in cotton-stoppered test tubes and stored at −18°C in a deep-freeze chest for from 63 to 118 days. On removal from storage, the tumor tissue was thawed at room temperature, minced, triturated, and finally, made up to a 3:1 suspension in 0.85% saline solution as outlined for the fresh accelerant. This suspension was homogenized by means of a Potter-Elvehjem homogenizer, and the homogenate was employed as the source of "frozen accelerant." The procedures for the preparation of accelerant factors from fresh unfrozen tissues and from frozen cells were carried out at room temperature. *Challenge inoculum.* The challenge inoculum consisted of a 5% suspension of viable Z8352 tumor cells which was prepared from freshly excised tumor by forcing the tumor tissue through a tissue press to yield a mince permeable for a 22-gauge needle, suspending this mince in a ratio of 1 g of minced tumor to 19 ml of 0.85% saline solution, filtering the suspension of tumor cells through 4 layers of 12-ply gauze and, finally, allowing the cellular filtrate in a 50 ml centrifuge tube to sediment for 10 minutes at room temperature. The resultant supernatant suspension of cells was transferred by pipette to another tube to be employed as the source of the challenge inoculum; the sediment was discarded.

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† The material contained in this paper will be submitted in partial fulfillment of the requirements for the degree of Master of Science at the University of Minnesota.

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Method of testing. The determination of the efficacy of each test material was carried out in two steps: (1) by the injection subcutaneously into the groin of a preparatory dose consisting of 0.2 ml of accelerant, whether frozen or fresh, and (2) by the challenge 10 days later of these test mice and of control mice that had not received a prior inoculation of either accelerant by the injection subcutaneously of 0.1 ml of a 5% suspension of viable Z8352 tumor cells into the tissues of the nape of the neck. The test and control recipient animals following challenge were observed every second day for the development of tumors and for the time of death; these findings were recorded.

Method of calculating results. Inasmuch as the transplantation of mouse mammary adenocarcinoma Z8352 cells into ZBC mice produces uniformly lethal results, the time required for the recipient animal to succumb to the challenge injection provided a precise and convenient reference point for the determination of acceleration of the neoplastic process. Accordingly, two methods were utilized for demonstrating the accelerative effect: (1) As a means of comparing the *relative longevity* of the control and test groups following challenge, a method for the determination of a "Percental Accelerant Index" was devised. This index was calculated from the formula:

[‡] The ZBC strain employed was developed in the following manner:

Ax ♀ (A strain lacking the milk agent) X Zb ♂ (Z or C_3H lacking the milk agent)
↓
AxZb F1 ♀ X Zb ♂
↓
ZBC

$$\text{Percental accelerant index} = \frac{\begin{bmatrix}\text{Interval in days required for}\\ \text{death of 50\% of control group}\end{bmatrix} - \begin{bmatrix}\text{Interval in days required for}\\ \text{death of 50\% of test group}\end{bmatrix}}{\text{Interval in days required for death of 50\% of control group}} \times 100$$

(2) As a means of comparing the *relative mortality* of the control and test groups during the course of the experiment, the percental mortality due to cancer within the control group at the time 50% of the test group animals were dead of cancer was determined; a similar determination was made of the percental mortality from cancer within the test group at the time 50% of the control group animals had succumbed to the challenge. As an aid for the reader in the interpretation of the data, the results of Exp. 1 of Series A (Table I) are cited as an example: Of the 14 test animals that received a prior injection of the frozen accelerant, 50% had died of cancer in 31 days, while only 10% of 17 control animals were dead at this time. Of the 17 control animals, 50% had died of cancer in 39 days, while 82% of the test animals were dead at this time. The calculated *Percental Accelerant Index* for this experiment was $\frac{39 \text{ days} - 31 \text{ days}}{39 \text{ days}}$, or 20% acceleration, due to the prior injection of the frozen accelerant.

Results. Two series of experiments were carried out to determine whether derivatives prepared from fresh and frozen cancerous tissues, respectively, would result in an enhancement in the rate and the extent of proliferative response of a challenge inoculum of homologous tumor cells which was injected subcutaneously after an interval of 10 days. This influence of the "accelerant" was determined by the accelerant index method described above.

Series A consisted of 3 experiments involving a total of 102 animals. This series was planned to determine whether enhancive factors for tumor growth as described by Casey (1-7), and others(13,17) could be elicited by the storage in the frozen state of mouse mammary cancer Z8352. That such enhancing factors do exist in this tumor is evident from the data presented in Table I. It can be seen from the findings presented in Table I that the prior inoculation of frozen accelerant had an accelerative effect of 22.2% (Percental Accelerant Index), as determined from a compilation of the results of Exp. 1, 2, and 3. Furthermore, when the "2 x 2 table" Chi square method(12) is applied to the comparative percental mortality of the *compiled* opposing groups, the results of these calculations establish for each of the compiled groups a significant P value of less than 0.01 (50% of 47 test mice vs. 17.4% of 55 control animals, $X^2 = 12.2$ and $P = 0.0005$; 50% of 55 control mice vs. 86.6% of 47 test animals, $X^2 =$

TABLE I. Effect of Frozen Accelerant on Growth of a Transplantable Mouse Mammary Cancer, Z-8352.

Series	Exp. No.	No. of animals by groups: Test	No. of animals by groups: Control	Deaths from cancer by groups as % incidence: Test	Deaths from cancer by groups as % incidence: Control	Accelerant index determination: Interval in days to calculation of % accelerant index	Accelerant index determination: % accelerant index
A	1	14	17	50	10	31	$\frac{39-31}{39} = 20$
				82	50	39	
	2	8	19	50	21	36	$\frac{47-36}{47} = 23$
				88	50	47	
	3	25	19	50	0	35	$\frac{51-35}{51} = 31$
				88	50	51	
	Compilation 1-3	47	55	50	17	35	$\frac{45-35}{45} = 22$
				87	50	45	

TABLE II. Effect of Fresh Unfrozen Accelerant on Growth of a Transplantable Mouse Mammary Cancer, Z-8352.

						Accelerant index determination	
Series	Exp. No.	No. of animals by groups Test	Control	Deaths from cancer by groups as % incidence Test	Control	Interval in days to calculation of % accelerant index	% accelerant index
B	4	21	9	50	0	37	$\frac{65-37}{65} = 43$
				93	50	65	
	5	12	17	50	6	30	$\frac{39-30}{39} = 23$
				75	50	39	
	6	22	19	50	16	42	$\frac{51-42}{51} = 17$
				86	50	51	
	Compilation 4-6	55	45	50	19	37	$\frac{49-37}{49} = 24$
				86	50	49	

15.3 and $P = 0.0001$).

Series B consisted of 3 experiments involving 100 animals. The purpose of this series was to determine whether an accelerating factor could be obtained from extracts of *fresh* (unfrozen) tumor tissue much as it had been derived from tumor tissue stored in the frozen state. That such an accelerant was obtained is evident from the data presented in Table II. The findings presented in Table II make it known that the prior inoculation of fresh (unfrozen) accelerant produced an accelerative effect of 24.5% (Percental Accelerant Index) upon the malignant process as determined from a compilation of the results of Exp. 4, 5, and 6. Again, when the "2 x 2 table" Chi square method is applied to the comparative percental mortality of the *compiled* opposing groups, the results of these calculations establish for each of the compiled groups a significant P value of less than 0.01 (50% of 55 test mice vs. 18.9% of 45 control animals, $X^2 = 10.4$ and $P = 0.0013$; 50% of 45 control mice vs. 86.6% of 55 test animals, $X^2 = 15.4$ and $P = 0.001$.

A graphic presentation of the *compiled* results of Exp. 1, 2, and 3 of Series A appears in Fig. 1, while a similar presentation of the *compiled* results of Exp. 4, 5, and 6 of Series B appears in Fig. 2.

Discussion. Studies were carried out which successfully demonstrated the presence of an accelerant factor in unfiltered supernatant fluid derived from fresh (unfrozen) mouse mammary carcinoma Z8352. This accelerant factor was made known in 3 experiments involving 100 ZBC mice which served as recipients for the transplantation of the tumor. Significantly, the results obtained with the accelerant factor derived from fresh tissue was corroborated by similar findings when tumor tissue which had been stored at —18°C for from 63 to 118 days was employed for the preparation of the accelerant or, "XYZ," factor.

The technic of an "accelerant index" for comparison of the test and control groups was introduced as a means for minimizing individual variations between animals within groups and at the same time to provide a convenient reference point for the comparison and compilation of replicate experiments. The validity of such a technic rests on the assumption that one of the most important criteria for assessing the extent of a malignant process is the rapidity with which a proliferating tumor produces death. It was recognized in this present study that the period of time required for the recipient animal to succumb to the transplantable tumor provided a precise and convenient reference point. This seemed preferable to the employment of periodic palpation, or volumetric measurements, of the growing tumor as an index of enhancement,

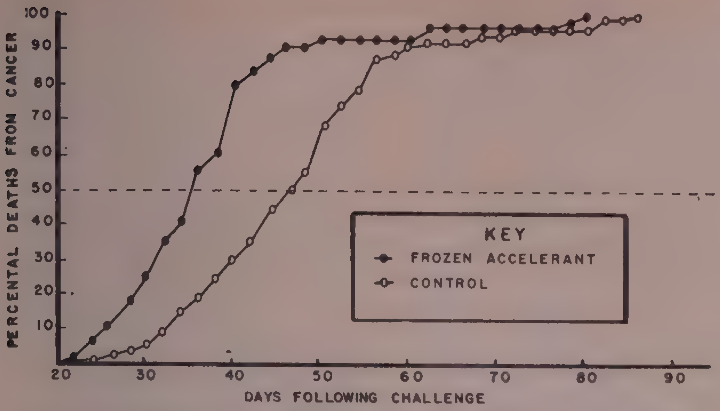

FIG. 1. The enhancement by frozen accelerant in the incidence and time to death: A graphic presentation of the compiled results of Experiments 1-3, Series A.

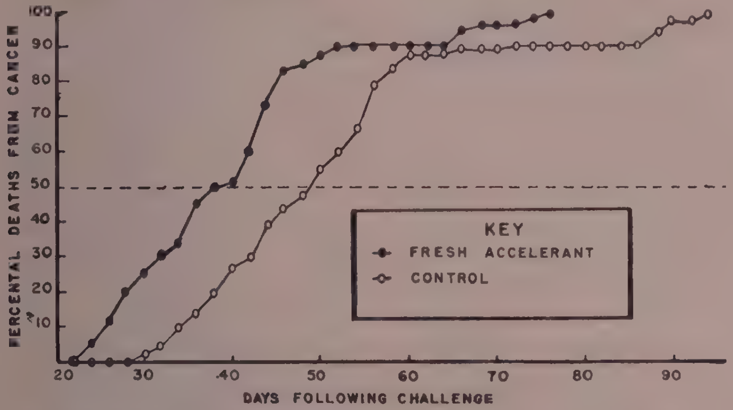

FIG. 2. The enhancement by fresh accelerant in the incidence and time to death: A graphic presentation of the compiled results of Experiments 4-6, Series B.

since these latter methods, regardless of the extreme care with which they may be conducted, are subject to interpretive differences when made by different observers, or even by the same person. Utilizing this technic, a significant acceleration in the onset of death (or a significant decrease in longevity) resulted when the tumor had been transplanted into test animals previously inoculated with either fresh (unfrozen) accelerant or frozen accelerant in comparison with control animals which had not been inoculated previously with either accelerant.

The striking similarity in the compiled results of the two series of experiments (Tables I and II and Fig. 1 and 2) strongly suggests that the two factors may in fact be a single entity derived from the tumor tissue by two different methods. In contrast to this evidence for acceleration, preliminary experiments indicate that the prior inoculation of mouse embryonic tissue, or fresh or frozen noncancerous lactating mouse mammary gland exerts little, if any, accelerating effect.

The possibility that the acceleration is not due to a specific phenomenon, but is merely the reflection of the summation effect of a double inoculum, may be discounted since in no instance did a tumor develop in the groin, the site which had been employed for the inoculation of the material under test. The only tumors arising occurred in the region of the nape, where the challenge inoculation of a 5% suspension of viable transplantable tumor cells was made. Furthermore, 10 animals inoculated only with frozen accelerant and 10 animals inoculated only with fresh (unfrozen) accelerant and allowed to remain unchallenged failed to develop tumors during a 90-day period of observation.

The findings that fresh (unfrozen) cancerous tissues and frozen tumor tissues yielded equally effective accelerative effects upon the growth of the homologous mouse mammary tumor are interpreted as evidence to relate the demonstration by Casey(1-7), and others (13,17), of accelerative factors in frozen tumor tissue and the occurrence of similarly reactive materials in lyophilized tumor tissue, as reported by Snell and his associates (14,15,20,21). Evidently, the accelerative, or "XYZ", factors originate in the tumor cell. It would seem that it is incidental whether the technics for eliciting enhancement employ fresh unfrozen tissues, lyophilized tissues, or frozen cells. However, in contrast to the currently reported unequivocal findings which made it known that fresh and frozen cells were equally productive of accelerative factors, it has been reported for mouse mammary carcinoma 15091a on transfer to C57 mice that the employment of fresh tumor homogenate resulted in an enhancement in only 28 of 62 (45%) mice(16) as compared with evidence for enhancement in 141 of 183 (77%) of the recipients that had been prepared by injections of lyophilized tumor tissues(15). It is suggested that the technic herein described for the preparation of accelerative factors will be equally productive when it is applied to mouse mammary cancer 15091a, irrespective of whether the tissues are employed as fresh, unfrozen cells or after lyophilization.

The failure of various investigators(8-11, 16,18,19) to demonstrate unequivocally the presence of accelerants in *filtrates* of fresh tumor tissue may have resulted, as suggested by Casey *et al.*(9), because "the factors are attached to or parts of a macromolecule which is not filtrable through Berkefeld or Seitz filters." The immunological, chemical, and physical properties, including filtrability, of the fresh (unfrozen) and the frozen accelerants derived from the Z8352 tumor, are under investigation in our laboratories.

Summary and conclusions. (1) In a series of 3 experiments involving 100 ZBC mice an accelerating factor was demonstrated in the unfiltered supernatant fluid which had been prepared by centrifugation repeatedly of a saline extract of fresh (unfrozen) mouse mammary carcinoma Z8352. This tumor, which originated in a female C_3H mouse possessing the milk agent, is maintained by transplantation in ZBC mice not possessing the milk agent. (2) In a second series of 3 experiments involving 102 ZBC mice a similar accelerant was demonstrated in homogenates of Z8352 tumor tissue which had been stored in a deep freeze chest at —18°C for from 63 to 118 days. (3) It is probable that the two factors, the "fresh (unfrozen) accelerant" and the "frozen accelerant", are a single entity derived from the tumor tissue by two different methods.

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Rate of Urinary Calcium Excretion Following Its Intravenous Administration as an Indicator of Bone Metabolism.* (19048)

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The purpose of this study is an attempt to define the state of bone metabolism and some of the mechanisms that maintain calcium and phosphorus homeostasis. The principle of the

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method is to alter calcium homeostasis acutely by the intravenous administration of calcium and to measure the serum levels of calcium and phosphorus and their renal excretion. The intravenous route eliminates the factor of variability in absorption of calcium from the gastro-intestinal tract. By performing these experiments under identical conditions in all cases, the results obtained in normals and in patients with various types and degrees of bone disease have been compared. While the present study has been performed under metabolic ward control and detailed data were obtained in order to define mechanisms of action, our aim is to evolve a simple and rapid test that will help define the state of bone metabolism at a given period.

Baylor, *et al.*, studied the fate of intravenously administered calcium in three individuals, a normal woman, a patient with postmenopausal osteoporosis, and a man recovering from partial starvation. The calcium retention was greatest in the latter patient, least in the woman with osteoporosis(1).

Method. 50 ml of 10% calcium gluconate solution, containing 446 mg of calcium, are diluted with 500 ml 5% glucose solution in water. The intravenous infusion is given after a 15-hour-fast at the constant rate of 60 drops per minute, so that the total infusion is administered in precisely 4 hours. After the completion of the infusion the patient is given a low calcium diet at regular meal times. Urinary samples are collected by means of an indwelling catheter for one hour before, and at ½, 1, 2, 3, 4, and 5 hours after the start of the infusion. The catheter is then removed and voided urinary samples are collected at 7, 9, 12, 24, and 48 hours after the start of the infusion. By means of a Cournand needle in the femoral artery, blood samples are collected before the infusion and at ¼, ½, 1, 2, 3, and 4 hours after the start of the infusion. The Cournand needle is then removed and subsequent blood samples are obtained by venopuncture at 5, 7, 9, 12, 24, and 48 hours after the start of the infusion. In order to correlate the results of the tests with clinical and metabolic data, the patients are observed on the metabolic ward on a low calcium diet prior to and following the acute experiment. The metabolic and chemical methods employed are described in a previous communication(2).

Results. Twenty adult patients have been studied; one patient was studied twice, another on 4 occasions. The series includes 4 normals and 16 patients with senile osteoporosis, osteomalacia secondary to idiopathic steatorrhea, multiple myeloma, breast carcinoma with osteolytic metastases, prostatic carcinoma with osteoblastic metastases, and cancer patients without clinical or roentgenographic evidence of bone involvement. The procedure caused no significant side reactions; this may be attributed to the slow rate of infusion. Renal function as measured by urea and creatinine clearances, showed no significant change during and after the infusion.

From the data obtained, the cases may be divided into 4 groups based on the urinary calcium excretion per 24 hours after the start of the infusion, (Fig. 1). Group 1, comprised normals, and patients with no demonstrable bone disease; the excretion rate was 302 ± 24 mg/24 hr. In Group 2, the excretion rate

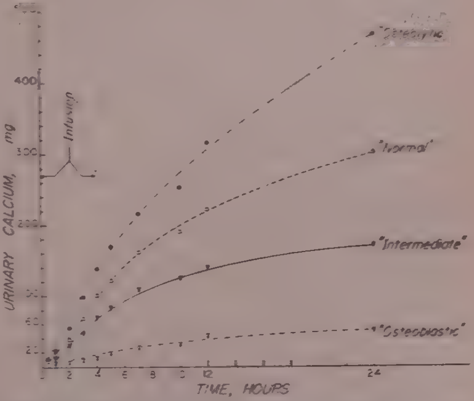

FIG. 1. The urinary calcium excretion during the 4 hr period of intravenous calcium infusion and during 20 hr thereafter. The values represent average cumulative excretion in 20 patients.

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2. Laszlo, D., Schulman, C. A., Bellin, J., Gottesman, E. D. and Schilling, A., *J.A.M.A.*, 1951, in press.

was 50 ± 14 mg/24 hr, indicative of an abnormally great tendency to calcium retention, presumably because of increased bone deposition. In Group 3, comprising patients with osteolytic lesions, the excretion rate was 469 ± 92 mg/24 hr, reflecting their decreased ability to utilize the administered calcium. In Group 4, "intermediate" in character, the rate of calcium excretion was 170 ± 31 mg/24 hr. This group comprised patients who had bone disease in the phase of bone repair, or greater bone formation than one would expect to find in normal adult individuals.

A good correlation has been noted between the calcium and phosphorus metabolic data and the calcium excretion following intravenous infusion, suggesting the usefulness of this method as an adjunct for the metabolic definition of the functional state of the skeletal system. From the data it appears that these excretion rates reflect fluctuations in bone repair earlier and more accurately than do roentgenographic and metabolic studies of the skeletal system. Fig. 2 and 3 illustrate the responsiveness of this method to the fluctuations in bone metabolism induced by therapy or occurring spontaneously. In patient 1, (Fig. 2) the first study was performed at a time when stilbestrol induced a clinical and metabolic remission of an advanced prostatic carcinoma(3). The patient at this time was in metabolic calcium equilibrium on a low calcium diet and the urinary calcium excretion was 17 mg/24 hr. The tendency toward bone deposition was reflected in the subnormal rate of calcium excretion in the acute experiment as illustrated in the lower curve. A second study was performed 5 months after the cessation of therapy, when both clinical and metabolic exacerbation of his disease were evident. At this time the calcium balance was markedly negative and the 24-hour urinary calcium excretion in the metabolic study had risen from 17 mg to 187 mg. Correspondingly, increased rates of calcium excretion were noted in the acute experiment. The reproducibility of the data was studied at this time; on 3 subsequent occasions, when no metabolic fluctuations were observed, similar rates of excretion were found.

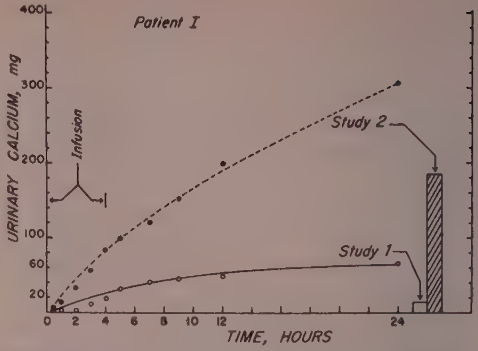

FIG. 2. Urinary calcium excretions on a low calcium diet (bar graph) and following intravenous calcium infusion (curve) in a patient with osteoblastic metastases secondary to prostatic carcinoma. Study 1 was performed during an estrogenic-induced remission. Study 2 was performed during relapse following estrogen withdrawal.

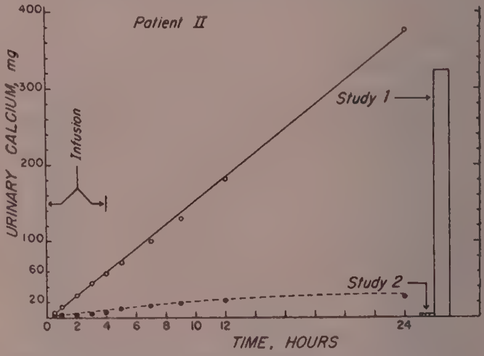

FIG. 3. Urinary calcium excretions on a low calcium diet (bar graph) and following intravenous calcium infusion (curve) in a patient with osteolytic metastases secondary to breast carcinoma. Study 1 was performed during an osteolytic crisis with hypercalcemia. Study 2 was performed during a spontaneous remission.

In patient 2, (Fig. 3), carcinoma of the breast with osteolytic metastases, the first study shows a phase of abnormally great bone dissolution. This is evidenced by high urinary calcium excretion during metabolic studies, 324 mg/24 hr, as well as by high rates of calcium excretion upon the intravenous administration of calcium. In the second study one month later, a phase of spontaneous bone repair was evidenced by a urinary calcium of 5 mg/24 hr during metabolic studies, a positive calcium balance, and

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low rates of excretion in the acute experiment.

Renal clearance of calcium and phosphorus are being investigated. Ca^{45} is being used as a tracer for the exogeneously administered calcium. These studies are in progress and will be reported in a subsequent communication.

Summary. The rate of excretion of calcium was studied following its intravenous infusion in normals, and in patients with various bone diseases. Subnormal rates were found in patients with an increased tendency to bone deposition. Increased rates were found in patients with osteolytic activity. The good correlation between these data and metabolic balance studies suggests the usefulness of this test in defining the state of skeletal activity and the effects of therapy thereon.

The authors wish to express their appreciation for the assistance given by Judith Bellin, Estelle D. Gottesman, and Vernice Vankinscott, of the Metabolic Unit.

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Encephalitis in Midwest VI. Western Equine Encepalomyelitis Virus Isolated from *Aedes dorsalis* Meigen. (19049)

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This paper reports the isolation of Western Equine Encephalomyelitis virus (WEE) from each of two batches of *Aedes dorsalis* (Meigen). These viruses were recovered during a study in Weld County, Colo., in 1950 in which specimens of 7 species of mosquitoes were routinely tested for neurotropic viruses. Natural infection of *Aedes* mosquitoes by a neurotropic virus was first reported by Hammon, Reeves, and Galindo(1) with 2 recoveries of western equine encephalomyelitis (WEE) virus from *Aedes dorsalis* (Meigen) in the San Joaquin Valley, Calif., in 1943. The previous year, Hammon and associates (2) failed to recover virus in tests of 4,866 *A. dorsalis* and 2,637 other *Aedes* mosquitoes collected in the Yakima Valley, Washington during an outbreak of encephalitis. On the other hand, WEE virus was isolated 41 times from 9,466 *Curlex tarsalis* Coquillette collected simultaneously with the *Aedes*. Furthermore, Hammon and Reeves(3) state that in tests of 25,162 *Aedes* mosquitoes from 5 western, midwestern, and southwestern states, the western equine virus was isolated only twice in contrast with an infection rate for *C. tarsalis* of 1 of each 225 tested during the 3-year period. It is possible that the vectors and hosts of W.E.E. virus in the midwestern region differ from those in the hot valleys of California. However, only four isolations of virus so far have been reported from mosquitoes in the midwest, three in North Dakota(4,5) and one in Nebraska(6); all were from *C. tarsalis*. The isolations of virus from *A. dorsalis* reported in this paper are the first from the midwest area.

Technics. The mosquitoes were captured by aspirator tube in natural resting places or

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TABLE I. Results of Tests of Mosquitoes for Viruses, Weld County, Col., May-August, 1950.

Mosquito species	No. tested	Neurotropic viruses isolated WEE
Culex tarsalis	1141	0
Culiseta inornata	197	0
Aedes dorsalis	4492	2
vexans	1928	0
cataphylla	64	0
sticticus	57	0
trivittatus	6	0
Total	7885	2

while attempting to bite, anesthetized lightly with ether, separated by species into ampoules, the ampoules sealed in an acetylene flame, and the still living specimens frozen on dry ice. Ten to 50 mosquitoes were sealed in each ampoule and sent frozen to the CDC Virus and Rickettsial Section, Montgomery, Ala., for test. In the laboratory the mosquitoes were triturated in a sterile mortar with buffered saline containing 30% by volume of inactivated normal rabbit serum, diluted to a volume of 1 to 3 ml depending on the number present, and the suspension centrifuged in a cold room at 13,000 rpm in an angle centrifuge. The supernatant fluid was then inoculated both intracerebrally (0.02 ml) and intraperitoneally (0.04 ml) into 12- to 14-day-old white mice. The brains of mice which died or showed symptoms of encephalitis were removed, tested for bacteria, and suspensions of them inoculated intracerebrally into fresh mice. Finally, the isolated viruses were identified serologically by cross protection tests.

Results. The study in the summer of 1950 in Weld County, Colorado, resulted in the collection and testing of a total of 7,885 mosquitoes, comprising 7 species as shown in Table I. All the mosquitoes were captured between May 2nd and August 29th.

The viruses isolated from each of 2 ampoules of *A. dorsalis* had the following characteristics: Each strain passed through a Seitz filter pad; was free from bacteria; was lethal to mice in dilutions of $10^{-6.5}$ to $10^{-8.5}$; and was neutralized by an antiserum of western equine encephalomyelitis virus, but not by that of eastern equine encephalomyelitis or the St. Louis encephalitis viruses. The identification of the virus strains as those of western equine encephalomyelitis was completed by the challenge inoculation of guinea pigs previously immunized against this virus.

Among the mosquitoes identified throughout the summer as *A. dorsalis,* it was noted that considerable numbers of a dark form appeared which were readily distinguishable, morphologically, from the normal *A. dorsalis.* The dark form of *A. dorsalis* has also been noted in California(7) and one of the 2 lots found infected with WEE virus consisted entirely of the dark form. The dark form was routinely placed in ampoules separate from the normal *A. dorsalis.*

Summary. 7885 mosquitoes of seven species were collected in Weld County, Colo., in 1951 and tested for neurotropic viruses. Two strains of western equine encephalomyelitis virus were recovered from 2 lots of *Aedes dorsalis.*

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Encephalitis in Midwest VIII. Neutralizing Antibodies in Sera of Small Wild Mammals. Colorado 1950. (19050)

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A series of exploratory investigations were carried out by the Communicable Disease Center in Weld County, Colorado, in 1950, in an attempt to find a promising lead which might result in the clarification of the ecology of the viruses of western equine encephalo-

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myelitis (WEE) and St. Louis encephalitis (St. Louis) in nature. The results of one of these limited studies, which was undertaken to evaluate the part played by the small mammals, particularly those belonging to the order Rodentia, will be reported here. Studies carried out on this group of animals in the past 10 years have shown that although there is evidence of past infection, the percentage of animals affected is less than that found in birds or domestic animals. In California in 1941, Hammon and his colleagues(1) examined sera from 12 species of wild animals for neutralizing antibodies and they found that less than 10% were positive against either WEE or St. Louis viruses. At the same time, domestic birds gave evidence of almost 50% infection against each of the viruses, and wild birds were approximately 30% infected. Howitt and Herrick(2), in an endemic area, found that practically 100% of the domestic animals were positive against the western virus and 60% against the St. Louis virus, while with sera from the wild mammals in the same area the positives were again less than 10%. This was confirmed in another study by Hammon *et al.*(3), wild animals being again less than 10% positive against each of the viruses, while domestic animals and birds were approximately 35% and 50% positive, respectively. However, the virus itself had been recovered once from a ground squirrel(4), and possibly (again) on a second occasion; and there are indications that St. Louis virus can live in the brain of a field mouse (*Reithrodontomys megalotis*) for as long as 10 days without showing evidence of infection(5). In the Argentine, a disease causing high mortality in young nutria (*Myocaster coypus*) has been recognized since 1944, and recently a virus of the equine encephalomyelitis group has been recovered from these rodents(6). In addition, rodents have been suggested as the possible natural reservoir for the "encephalomyocarditis" group of viruses(7). On the other hand, WEE virus had been recovered only once from a bird in nature(8), and St. Louis virus had not been recovered at all.

In Weld County, Colorado, in 1949, during a small epidemic of WEE virus infection in humans and horses, the Center conducted a brief survey of the antibodies of WEE and St. Louis viruses in the bird population and found that out of 100 wild and domestic bird sera tested, approximately 30% gave positive reactions against WEE, but all were negative against the St. Louis virus(9). The present study consisted of testing sera from a wide range of small wild mammals in Weld County for neutralizing antibodies to these viruses to see if the percentage of positives would equal or exceed that of the birds. In such an event, it was planned to extend the studies and attempt to isolate the virus from the small mammals. However, if 10% or less were positive, as found in California, it would be concluded that the role of these mammals as a permanent reservoir of the viruses would be less than that of the birds, and further efforts would be concentrated on the birds rather than mammals.

Methods. Small mammals were trapped alive by all available methods, bled, and their sera separated. The specimens were sealed in tubes and stored in dry ice until shipment could be undertaken to the Center's virus and rickettsia laboratories in Montgomery, Ala. The specimens were packed in dry ice and shipped by air express. The technic of calculating the neutralization index was that

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TABLE I. Weld County, Colo., 1950. Small Mammal Sera. Tested for neutralizing antibodies against the viruses of Western Equine Encephalomyelitis and St. Louis Encephalitis.

Species	No. tested WEE	No. WEE positive	No. tested St. Louis	No. positive St. Louis
Rodents				
Perognathus hispidus paradoxus (Kans. Pocket Mouse)	5		3	
Dipodomys ordii richardsoni (Richardson's Kangaroo Rat)	37	1	25	
Onychomys leucogaster arcticeps (Great Plains Grasshopper Mouse)	2		1	
Reithrodontomys megalotis dychei (Prairie Harvest Mouse)	1		1	
Peromyscus maniculatus osgoodi (Black-eared Deer Mouse)	6		4	
Microtus pennsylvanicus modestus (Sawatch Meadow Mouse)	5		3	
Rattus norvegicus (Norway Rat)	18		7	
Spermophilus tridecemlineatus pallidus (Pale-striped Ground Squirrel)	2		1	
Spermophilus spilosoma obsoletus (Northern Spotted Ground Squirrel)	4		1	
Cynomys ludovicianus (Black-tailed Prairie Dog)	26		15	
Scirus niger rufiventer (Western Fox Squirrel)	6	1	5	1
Other mammals				
Myotis volans interior (Interior Long-legged Bat)	2			
Procyon lotor (Raccoon)	5		3	
Mephitis mephitis varians (Long-tailed Texas Skunk)	3		3	
Taxidea taxus taxus (Badger)	1		1	
Lepus townsendii campanius (White-tailed Jack Rabbit)	6	1	5	
Lepus californicus melanotis (Great Plains Jack Rabbit)	24	2	14	
Lepus americanus bairdii (Rocky Mt. Snowshoe Rabbit)	1		1	
Sylvilagus sp. (Cottontail)	37		16	
Total	191	5	109	1

given in the U. S. Army laboratory manual. All sera were inactivated at 56°C for 30 minutes. The serum virus mixtures were prepared in the late afternoon and held overnight in the refrigerator before the inoculation of the mice. Indices of 50 or more were regarded as positive. Results of the survey are given in Table I.

Discussion. It has been pointed out frequently by workers on yellow fever that results of serum surveys in wild birds and mammals are most difficult to evaluate. Certain of the sera from these animals possess non-specific viricidal substances which can give false positives in the neutralization tests. Moreover, neutralizing antibodies develop in some of the animals, following a viremia so transient as to prevent these animals from taking any prominent part in the permanent maintenance of the infection in nature. In other animals the antibodies tend to disappear very rapidly, so that a negative result may not necessarily mean that the animal in question has never been infected.

With these points in mind, it may be concluded from our figures that the percentage of positives found in the survey of mammals is well below the percentage for birds. These findings give little encouragement for the continuance of further work on this type of animal life.

Summary. (1) A serum survey of small mammals for neutralizing antibodies showed that 2.6% were positive against WEE virus and less than 1% against St. Louis virus. (2) In view of the finding some months earlier that wild and domestic birds gave 30% positives, it was concluded that further study of the small mammals was less likely to be productive of major results than the study of birds.

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Intraperitoneal Susceptibility of Suckling Mice to MEF1 Poliomyelitis Virus.* (19051)

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Casals and Olitsky have recently adapted the MEF1 strain (Lansing type) of poliomyelitis virus to suckling mice, and have demonstrated that a specific complement fixing antigen may be produced from the CNS of these animals(1-3). The present communication reports the results of experiments designed to determine the susceptibility of suckling mice to the intraperitoneal inoculation of this adapted MEF1 strain. The positive results obtained suggest the possibility that this may provide a useful and sensitive method of determining the antibody content of various agents used to confer passive protection. Preliminary experiments employing gamma globulin tend to bear this out.

Materials and methods. The suckling mouse adapted strain of MEF1 virus (54th passage) was made available to us through the kindness of Dr. Casals and was maintained in our laboratory by intracerebral passage in 3-day-old mice which were sacrificed when moribund or paralyzed. The brains and spinal cords were harvested and pooled; the titer of the resulting suspensions varied from $10^{-3.5}$ to $10^{-4.4}$ when titrated in 3-4-week-old mice

TABLE I. Mortality of Mice of Various Ages Inoculated with .05 ml or .1 ml of 10^{-1} Virus Dilution by the Intraperitoneal Route.

	Mortality ratio of mice						
Age, days	Exp. 1	2	3	4	5	Total	Total % mortality
1	2/16*					2/16	13
2	1/7	2/12				3/19	16
3	1/5	9/22				10/27	37
5	8/11	5/20	22/38	20/30		80/160	50
6	6/15	19/46					
7	7/12	3/5				10/17	59
8	3/4					3/4	—
9		4/9				4/9	44
13					5/9	5/9	56
15					3/3	3/3	—
16					6/19	6/19	32
19					5/20	5/20	25
21–28	1/32	0/16				1/48	2
Mouse passage	60	54, 56, 60	59	59	60		
I.P. inoculum (ml)	.1†	.05	.1	.1	.1		
I.C. titer		$10^{-4.4}$	$10^{-3.7}$	$10^{-3.5}$	10^{-4}		

* Numerator/denominator = No. deaths/No. inoculated.

† Mice 1 and 2 days old given only .05 ml of virus suspension in Exp. 1.

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* Aided by a grant from the National Foundation for Infantile Paralysis.

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TABLE II. Intraperitoneal Titration of MEF_1 Virus in Mice 6 Days of Age.

Virus dilution	Mortality ratio of mice			Total % mortality
	Exp. 1*	Exp. 2†	Total	
10^{-1}	11/12	20/30	31/42	74
10^{-2}	2/12	0/6	2/18	11
10^{-3}	1/12	—	1/12	8.3
10^{-4}	2/12	—	2/12	16.7

* Intracerebral titer in 3-4-week-old mice: $10^{-4.4}$; suckling mouse passages 54, 56, 60, pooled. Intraper. inoculum: .05 ml.

† Intracer. titer in 3-4-week-old mice: $10^{-3.5}$; suckling mouse passage 59. Intraper. inoculum: .1 ml.

by the intracerebral route of inoculation. The CFW strain of Swiss mice was employed. Mice of various ages (suckling and weaned) were inoculated intraperitoneally with 0.05 or 0.1 ml of a 10% suspension of virus, and were observed for paralysis and death over a 21-day period. Considerable variability in the susceptibility of individual litters was noted in the first experiment; this was partially controlled in later ones by pooling all the suckling mice used in a given experiment and redistributing them at random to the mothers.

Results. Table I presents the results of 5 experiments testing the susceptibility of mice of various ages to intraperitoneal inoculation. Although mice 1 to 3 days old were comparatively resistant the fatality rate in animals from 4 to 14 days of age inclusive varied from 50 to 75%. Beyond the age of 15 days susceptibility fell off again. The majority of deaths occurred between the 4th and 10th days after inoculation, regardless of the age of the mouse.

In Table II are presented the results of 2 titrations carried out by the intraperitoneal route. Only scattered irregular deaths occurred in mice inoculated with dilutions higher than 10^{-1}.

Summary. Suckling mice 4-15 days old inclusive are susceptible (50-75% mortality) to the intraperitoneal inoculation of a 10% brain-cord suspension of the suckling mouse adapted MEF1 strain of poliomyelitis virus. Considerable variation in the susceptibility of individual mice of a given age was observed. The possible usefulness of this technic in passive protection studies employing gamma globulin or other agents is pointed out.

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Effect of Anti-histaminics on Experimental Nephritis in Rabbits. (19052)

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Acute nephritis may be produced in rabbits by the injection of duck serum containing antibodies against rabbit kidney. This reaction was first studied by Masugi(1,2). The exact mechanism is not clear. The antibodies of the anti-rabbit-kidney serum (NTS) are fixed promptly in the kidneys(3), nephritis developing after a latent period of 6-8 days. Kay(4,5) demonstrated that the onset of nephritis was coincident with the appearance of antibodies against duck serum.

Reubi(6,7) has reported the successful modification or prevention of this pathological change by the administration of anti-histaminics.

Methods. Dutch type, 1-2 kg rabbits and white domestic ducks were used. Ducks were injected twice a week for 10 weeks with 5 ml of a 10% suspension of homogenized rabbit kidney. Pooled sera had a precipitin titer of 1/16 against rabbit kidney.

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* Present address: Dept. of Physiological Chemistry, Yale University School of Medicine, New Haven, Conn.

† Present address: Dept. of Pathology, City Hospital, Cleveland, O.

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Fourteen rabbits received 6.6 ml of NTS I.V., 7 were treated with N.N. dimethyl N benzyl N. (alpha pyridyl) ethylene diamine (Pyribenzamine) and 7 served as controls. Five rabbits received 6.6 ml normal duck serum. The treated group received 15 mg/kg Pyribenzamine subcutaneously every 6 hours for 4 days, followed by 10 mg/kg every 4 hours until they were killed 14 days after the initial injection.

A second series of 28 rabbits received 5 ml of NTS. Fourteen received 0.8 mg Pyribenzamine subcutaneously every 3 hours starting 2 hours before the injection of NTS. They were sacrificed at the end of 8 days.

Urine was collected and examined for protein and formed elements each day. Terminally, the kidneys were examined histologically.

Results. Heavy albuminuria, hematuria, cylindruria appeared in all rabbits receiving NTS. The onset of albuminuria was delayed 1-3 days in those receiving Pyribenzamine in both series. Histologically, severe glomerular and tubular damage was present in all animals receiving NTS. No difference in severity was noted between those treated with Pyribenzamine and the untreated controls.

These results are in contrast with those of Reubi(6,7) who reported modification of nephritis using "Antistine" 50 mg subcutaneously twice daily and 75 mg 4 times daily, with diminution of albuminuria and striking improvement histologically. He concluded that histamine or "H substance" was of primary importance in the pathogenesis of nephritis, both experimental and in man. No evidence for a role of histamine in this tissue-damaging, antigen-antibody reaction was obtained in this experiment.

Summary. Acute nephritis was produced in rabbits by the injection of duck anti-rabbit-kidney serum. Attempt to modify the reaction by the intensive administration of Pyribenzamine was ineffective as judged by the histological picture, although it was accompanied by a slight delay in the onset of albuminuria.

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Simultaneous Occurrence of Two Immunological Types of Group A "Coxsackie" Virus in a Case of Herpangina. (19053)

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(Introduced by Karl Habel.)

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We are reporting the isolation of 2 Coxsackie viruses present simultaneously in the same individual because it represents an unique situation arising during our investigations of these agents(1,2). It has occurred in only one of the 106 persons from whom we have isolated Group A Coxsackie viruses during the past 2 years.

Experimental. The 2 viruses were isolated from L.B., a 2-year-old Negro male, ill with herpangina, who was seen at the outpatient department of Children's Hospital, Washington, D.C., in August 1950. He was first seen 3 days following his onset of illness, at which time an acute stage blood specimen was drawn. A fecal specimen was obtained 4 days later and a convalescent blood specimen was drawn 15 days following the onset of illness.

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1. Huebner, R. J., Armstrong, C., Beeman, E. A., and Cole, R. M., *J.A.M.A.*, 1950, v144, 609.

2. Huebner, R. J., Cole, R. M., Beeman, E. A., Bell, J. A., Peers, J. M., *J.A.M.A.*, 1951, v145, 628.

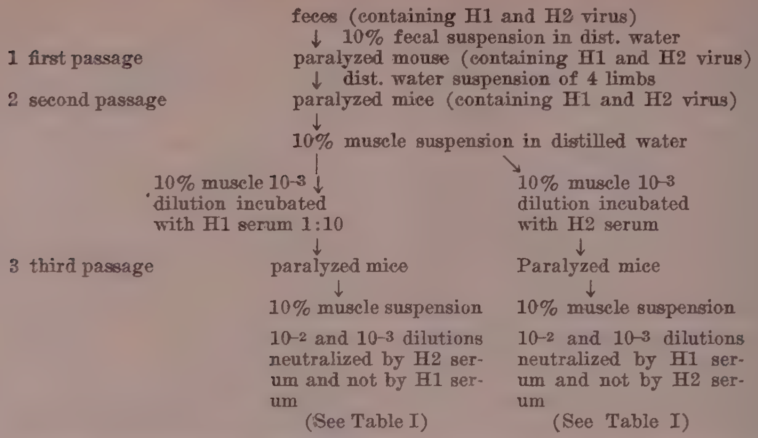
feces (containing H1 and H2 virus)
10% fecal suspension in dist. water
1 first passage
paralyzed mouse (containing H1 and H2 virus)
dist. water suspension of 4 limbs
2 second passage
paralyzed mice (containing H1 and H2 virus)
10% muscle suspension in distilled water
10% muscle 10^{-3} dilution incubated with H1 serum 1:10
10% muscle 10^{-3} dilution incubated with H2 serum
3 third passage
paralyzed mice
Paralyzed mice
10% muscle suspension
10% muscle suspension
10^{-2} and 10^{-3} dilutions neutralized by H2 serum and not by H1 serum
10^{-2} and 10^{-3} dilutions neutralized by H1 serum and not by H2 serum
(See Table I)
(See Table I)

FIG. 1. Flow chart demonstrating steps in the separation of H1 and H2 viruses from the fecal specimens of patient L. B.

The detailed methods used for virus isolations, neutralization tests, and complement fixation technics will appear later(3) and only a brief description of methods will be given here. A centrifuged, ether treated, distilled water suspension of the fecal specimen inoculated into a litter of 4-day-old albino suckling mice produced the characteristic paralysis associated with the histopathological lesions of Group A Coxsackie virus infection in all the mice within 4 days. A suspension made in distilled water from the limb muscles of one of the above mice was used to infect several litters of 4-day-old mice. Following the onset of paralysis in the latter, muscle tissue was harvested from the limbs and an ether treated 10% suspension in distilled water was prepared representing second passage virus. A preliminary examination of second passage virus by complement fixation with a group of reference immune serums indicated that the suspension probably contained the H_2 immunological type of Group A Coxsackie virus(1-3). For definitive immunological classification by the neutralization technic, serial 10-fold dilutions of the second passage suspension were incubated with H1, H2, and H3 Group A serums diluted 1:10 and the mixtures were inoculated into litters of suckling mice. The mice inoculated with the virus-H_2 serum mixtures survived several days longer than mice infected with either the comparable dilutions of the virus titration, or the virus mixed with the H1 and H3 serum; however, the mice inoculated with the virus-H2 serum mixtures eventually developed paralysis and died within the test period. A repetition of the neutralization test with the same materials yielded identical results. It then seemed probable that the second passage suspension contained more than one virus. A 10^{-3} dilution of this suspension was incubated with H2 serum diluted 1:10 and the mixture was inoculated into several litters of suckling mice. Limbs were harvested from the mice that became paralyzed from this inoculum and a 10% ether treated muscle suspension was prepared. Complement fixation testing of the latter suspension with a battery of Group A reference immune serums indicated the probable presence of H1 virus.

As a check, aliquots of a 10^{-3} dilution of the second passage virus were incubated separately with H1 and H2 immune serums and several litters of mice were inoculated with each mixture. Ten per cent muscle suspensions representing third passage virus were subsequently made from the mice paralyzed as a result of inoculation with these mixtures. Both third passage virus suspensions were tested by neutralization with H1 and H2 immune serums in suckling mice and each sus-

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3. Beeman, E. A., Huebner, R. J., and Cole, R. M., *Am. J. Hygiene,* in press.

TABLE I. Results of Neutralization Tests in Suckling Mice of 2 Series of Third Passage Muscle Virus Suspensions Demonstrating Separation of Viruses Originally Present in Feces of L. B.

	Virus dilutions											
	H1 serum			H2 serum			Virus titration				Log serum neutralization index vs. homologous virus	Log serum neutralization index vs. heterologous virus
Virus	10^{-2}	10^{-3}	LD_{50}†	10^{-2}	10^{-3}	LD_{50}	10^{-5}	10^{-6}	10^{-7}	LD_{50}		
1st series												
Third passage suspension derived from second passage virus—incubated with H1 serum	8/8*	8/8	>3.5	0/8	0/8	<1.5	7/7	8/8	5/8	>7.2	>5.7	<3.7
Third passage suspension derived from second passage virus—incubated with H2 serum	0/7	0/8	<1.5	8/8	7/7	>3.5	8/8	5/8	1/8	6.3	>4.8	<2.8
2nd series												
Third passage suspension derived from second passage virus—incubated with H1 serum	8/8	8/8	>3.5	0/8	0/8	<1.5	8/8	8/8	3/8	>6.8	>5.3	<3.3
Third passage suspension derived from second passage virus—incubated with H2 serum	1/8	0/7	1.6	8/8	8/8	>3.5	8/8	8/8	4/8	7	5.4	<3.5

Serial 10-fold dilutions of virus suspension incubated 2 hr at 37°C with equal amounts of immune serum diluted 1:10. Following incubation, mixtures placed in cold water bath at 4°C and each mixture inoculated into litter of 4-day-old suckling mice. Inoculum consisted of .04 ml of mixture given by the intraperitoneal route.

* No. died/No. inoculated.

† 50% infectious doses causing death or paralysis. Reed-Muench method.

pension showed evidence of only one virus corresponding to the type not neutralized by the immune serum incubated with the second passage virus inoculum. The separation of the viruses is shown in the flow chart in Fig. 1.

Having established the presence of 2 viruses in the second passage muscle suspension, it was of importance to determine whether accidental contamination had occurred in making the serial passage from the mouse paralyzed by the fecal inoculum. A fresh suspension made from a different portion of the original fecal specimen was inoculated into a litter of 4-day mice. When paralysis occurred a muscle suspension was made from one paralyzed mouse and was inoculated into several litters of 4-day mice. The muscle suspension from the latter, representing second passage virus was processed as above for the first series. Evidence was obtained again of two viruses, H1 and H2, by repetition of the procedures described for the first series of passages. Table I gives the results of neutralization tests performed with the third passage virus suspensions of both lines of virus derived from the same fecal specimen. The results indicate that the 2 viruses had been present in the original stool and had been separated in the 3rd passage by the reciprocal neutralization of each component in the 2nd passage suspensions with the appropriate immune serum.

An additional experiment was performed to demonstrate the presence of the H1 and H2 types in the feces. The original stool suspension and the second suspension used to recheck the results of the original were submitted to the following procedure: Aliquots of both suspensions were incubated with equal amounts of (1) normal mouse serum, (2) H1

TABLE II. Neutralization Tests* in Suckling Mice of Viruses Present in Original Fecal Suspension (10% in Distilled Water).

	Serums			
Fecal suspensions	Normal mouse serum	H1 serum	H2 serum	H1 and H2 serum
1st	6/6†	8/8	7/7	0/8
2nd	8/8	8/8	8/8	0/7

* See technic in Table I.

† Mice died/No. inoculated.

TABLE III. Immune Response of L. B. to Viruses Isolated From His Fecal Specimen and to Other Viruses.

Days serum obtained following onset of herpangina	Log of neutralization index of serum 1:10 against viruses isolated		Log of neutralization index of serum 1:10 against other Group A viruses	
	H1	H2	H3	H4
3	0	3.6	0	4.3
15	3.2	4.5	0	4.5

serum alone, (3) H2 serum alone, (4) H1 and H2 serum combined. The serums were used diluted 1:10. The mixtures were each inoculated into a litter of suckling mice. The results are shown in Table II. Paralysis and death occurred in all but the two litters inoculated with the fecal suspension-combined serum mixtures. It seems unlikely that the original fecal specimen could have been accidentally contaminated with another virus either in the laboratory or in the home.

Table III illustrates the serological response of the patient to the two viruses isolated from him as well as to 2 other Group A types. The results are expressed as the logarithm of the neutralization index of the serum diluted 1:10. The patient possessed neutralizing antibodies in his acute stage blood serum for the H2 virus followed by a slight rise in the convalescent serum. He possessed no detectable antibodies in the first serum against the H1 virus and his second serum showed the development of an appreciable level of antibodies against this type. It is probable that the patient's acute illness during which the fecal specimen was taken had been caused by the H1 virus. Since no history was obtained of a similar antecedent illness within one to 2 months prior to this episode, he may have been recovering from an inapparent infection produced by the H2 virus. The patient had antibodies against the H4 virus in both serums with approximately the same titer in each. This indicates prior infection with the H4 virus. It is not uncommon to find neutralizing antibodies in children against multiple types of Group A virus(3).

Discussion. In earlier reports(1,2), we have described 2 outbreaks of illness in a small Maryland community wherein 5 of the cases were infected with the Group A Coxsackie virus the first year followed by infection with a different immunologic type the succeeding year. Four of the 5 persons became ill with what is now regarded as herpangina caused by the Type 2 virus in 1949. The other was asymptomatic but harbored the virus in his feces. In 1950, 2 of the 5 had herpangina and the other 3 became infected without evidence of illness. The H3 virus was isolated from these persons at that time. In 1950 two viruses were present simultaneously within the same family group in the Washington, D.C area in different cases of herpangina(4). Based on 2 years study of isolations of Group A Coxsackie viruses from various population groups and illnesses (mainly herpangina) it is apparent that these viruses are widely prevalent during the summer and early fall months among the pediatric age group(4). There is no evidence at present to indicate that infection with one immunological type confers immunity to other known types of Group A viruses. It is therefore possible for a person to become infected in rapid succession with the virus types to which he is susceptible and to which he has had opportunity for exposure.

Summary. Two viruses occurring simultaneously were isolated from a case of herpangina. They were identified as the H1 and H2 immunologic types of Group A Coxsackie virus.

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4. Cole, R. M., Bell, J. A., Beeman, E. A., and Huebner, R. J., *Am. J. Public Health*, in press.

Received September 17, 1951. P.S.E.B.M., 1951, v78.

Inhibition Studies of Some Phosphoramides Against Sarcoma 180.[*] (19054)

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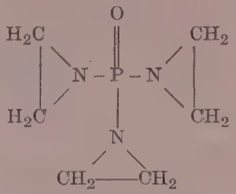
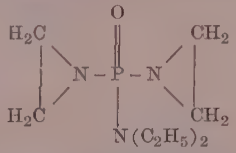
Recently, it has been shown that SK 1133, 2,4,6-tris(ethylenimino)-s-triazine, is quite effective in retarding the growth of a number of experimental tumors in mice and rats(1-7). SK 1133 has also been tried in over 150 cases of human cancer with beneficial results comparable to the nitrogen mustards and with some additional advantages(8).

Our studies with the ethylenimines have been extended by the synthesis of N-ethylene substituted phosphoramides and their subsequent testing for ability to inhibit the growth of various experimental tumors. The present report is concerned with the ability of two phosphoramides, SK 3818, N,N′,N″-triethylene-phosphoramide and SK 4614, N,N-diethyl-N′,N″-diethylene-phosphoramide, to inhibit the development of Sarcoma 180 in mice (Fig. 1). The relationship of dosage, toxicity, and retardation of tumor growth were determined and the picture of the peripheral blood and histopathological changes in the tumors and the organs of treated mice were studied.



I. SK 3818
N,N′,N″–triethylene phosphoramide



II. SK 4614
N,N–diethyl–N′,N″–diethylene phosphoramide

FIG. 1.

Materials and methods. The compounds were prepared and characterized by elementary analysis in the research laboratories of the Calco Chemical Division following the procedures of Bestian(9). In view of their high degree of reactivity the compounds were prepared as solutions with physiological saline just prior to injection. After preliminary toxicity determinations in mice(10) the compounds were tested by a standardized technic we have employed on over 5000 compounds(11) in seeking for anti-tumor activity. White Swiss mice (CFW Female and Male, RF Female and Male, 20 ± 2 g in weight) were used as experimental objects. They were implanted with Sarcoma 180 by the usual trocar method(12). The course of injections was begun 24 hours after implantation of the tumor. The compounds were adminis-

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[*] We wish to acknowledge support of this study by funds from the American Cancer Society to the Sloan-Kettering Institute for Cancer Research.

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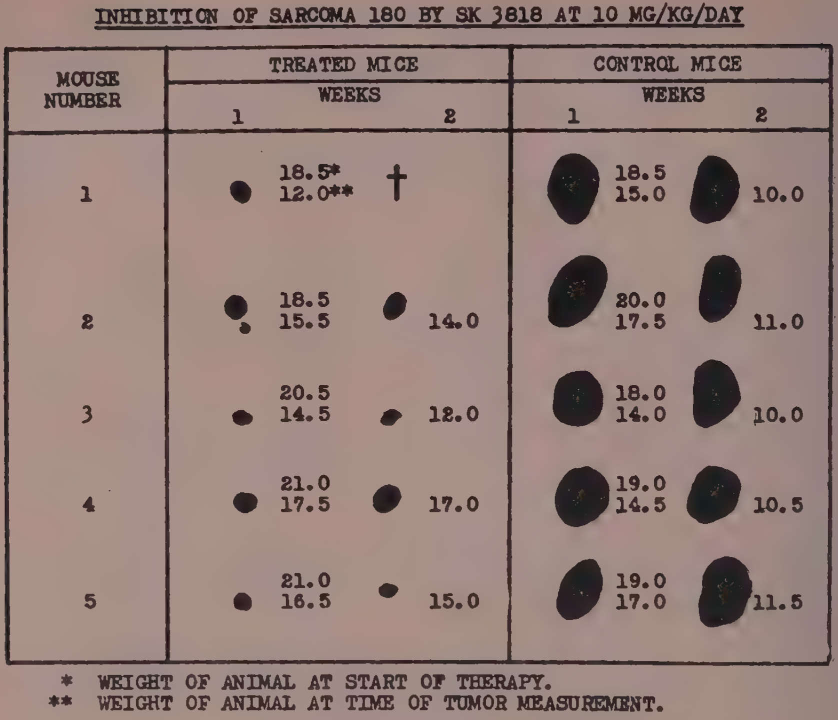


INHIBITION OF SARCOMA 180 BY SK 3818 AT 10 MG/KG/DAY

MOUSE NUMBER	TREATED MICE		CONTROL MICE	
	WEEKS		WEEKS	
	1	2	1	2
1	18.5* 12.0**	†	18.5 15.0	10.0
2	18.5 15.5	14.0	20.0 17.5	11.0
3	20.5 14.5	12.0	18.0 14.0	10.0
4	21.0 17.5	17.0	19.0 14.5	10.5
5	21.0 16.5	15.0	19.0 17.0	11.5

* WEIGHT OF ANIMAL AT START OF THERAPY.
** WEIGHT OF ANIMAL AT TIME OF TUMOR MEASUREMENT.

FIG. 2.

tered once daily intraperitoneally for seven days. Tumor bearing control mice were injected with corresponding amounts of physiological saline. On the eighth day after tumor implantation the tumors of the treated and of the control group were measured in two diameters by calipers. The degree of tumor inhibition was expressed as the ratio of the average diameter of the tumors of treated to those of untreated tumor bearing mice. The degree of inhibition of tumor growth was determined as follows: Inconclusive result (Inc.) = Three or more of a group of 5 mice succumbed during inhibition test. Marked Inhibition (†) = No growth of tumors in treated animals to a growth with an average diameter one-fourth that of the untreated controls. Slight Inhibition (‡) = Growth of tumors in treated animals from one-fourth to three-quarters the average diameters of the control tumors. No Effect (*) = Growth of tumors greater than three-quarters the average diameter of the control tumors.

Histopathological changes in tumors and organs of treated mice were studied in a special experiment as described previously (12). In brief, tumor pieces were implanted into the right thigh muscle of mice in groups of 8 each. Daily injections of the compounds (treated groups) and of physiological saline (control group) were started 4 days after tumor implantation and continued for 7 days. Three, 5 and 7 days after beginning of therapy mice in the different groups were sacrificed immediately after blood had been secured for counts. The tissues (tumor, thymus, spleen, axillary and mesenteric nodes, femur, sternum, stomach, intestine, lungs, heart, voluntary muscle, liver, both kidneys, adrenals and pancreas) were fixed in Zenker-formol solution and stained with hematoxylin-eosin for microscopic examination. Thus, peripheral blood and progression of possible lesions were studied 3, 5 and 7 days after beginning of therapy.

Results. 1. Relationship of dosage, toxicity, and retardation of growth of tumor. The degrees of retardation of tumor growth ob-

TABLE I. Relation of Dose to Inhibition of Sarcoma 180 by SK 3818.

Dose, mg/kg/day	No. of deaths*/ No. of mice	AWC	Tumor inhibition Grading	IQ × 100
25	3/5	—	Inc.	—
20	8/30	−4.5/−1.5	‡ to Inc.	30
15	6/45	−4.5/−1.5	‡ to †	25
12.5	4/35	−3.0/−1.0	‡	35
10	1/65	−4.0/−1.5	‡	40
8	0/20	−4.0/−1.5	‡	50
7.5	6/50	−3.0/−1.5	‡	40
6	0/10	−2.5/−2.0	‡	50
5	1/45	−2.5/−1.5	‡	55
4	0/10	−2.5/−2.0	‡	60
2.5	0/55	−1.5/−1.5	§ to ‡	75
2	0/30	0 /−1.0	§ to ‡	85
1	0/25	−1.0/−1.5	§ to ‡	85
.5	0/10	−1.0/−1.5	§ to ‡	90
.1	0/5	+2.0/ 0	§	80

* Results at end of one wk inhibition test.
† Marked inhibition. ‡ Slight inhibition. § No effect.

AWC = Avg wt change, as ratio of avg change per treated mouse to that per untreated control mouse.

IQ = Inhibition quotient = ratio of avg diameter of tumors in treated mice to those in untreated control mice.

Inc. = Inconclusive (3 or more out of 5 mice died).

TABLE II. Relation of Dose to Inhibition of Sarcoma 180 by SK 4614.

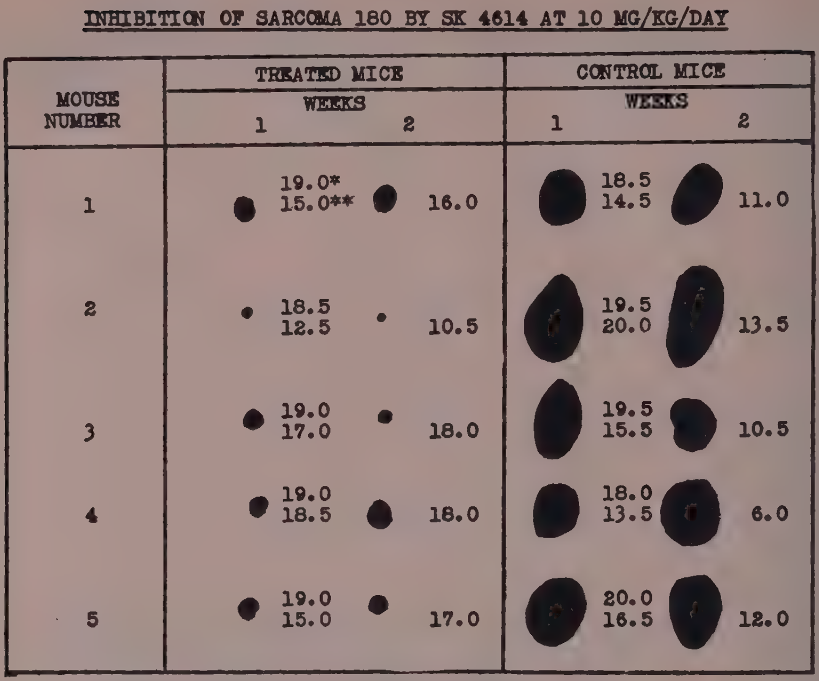
Dose, mg/kg/day	No. of deaths/ No. of mice	AWC	Tumor inhibition Grading	IQ × 100
20	7/30	−4.0/−2.0	‡ to Inc.	35
15	8/50	−3.5/−2.0	‡ to †	35
12.5	2/40	−2.5/−2.5	‡	20 to 35
10	1/60	−2.5/−1.5	‡	45
7.5	0/30	−1.5/−0.5	‡	55
5	1/40	−1.5/−1.0	* to ‡	65
3	0/40	−1.5/−1.5	* to ‡	70 to 80
2.5	0/10	−0.5/−0.5	*	90
2	0/20	−1.0/−1.5	* to ‡	75 to 85
1	0/15	0 /−0.5	*	90
.5	0/5	0 /−0.5	*	90
.1	0/5	−1.0/−0.5	*	90

* No effect. † Marked inhibition. ‡ Slight inhibition.

AWC = Avg wt change, as ratio of avg change per treated mouse to that per untreated control mouse.

IQ = Inhibition quotient = ratio of avg diameter of tumors in treated mice to those in untreated control mice.

tained at various levels of SK 3818 and SK 4614 are presented in Tables I and II. Both compounds have the ability to inhibit the growth of Sarcoma 180. The grading of a marked inhibition was observed in both instances at a dose level of 15 mg/kg/day for 7 days. The relationship of dosage, acute and delayed toxicity and degree of tumor inhibition appeared to be optimal at a dose level of 10 mg/kg/day for 7 days. Fig. 2 and 3 present area diagrams of the relative size of tumors of representative treated and untreated mice as observed at the end of the inhibition test and one week after therapy had been discontinued. Both compounds have similar inhibitory effects at the dose level employed. A comparison of the activity range, however, indicates that SK 3818 is characterized by a somewhat greater range of effective dose levels. SK 4614, for example, shows approximately a 5-fold range between the minimum effective dose and the maximum dose giving a conclusive result, whereas SK 3818 has a corresponding range of approximately 6. SK 1133, 2,4,6-tris(ethylenimino)-s-triazine, as discussed in a previous study(13), showed a



INHIBITION OF SARCOMA 180 BY SK 4614 AT 10 MG/KG/DAY

MOUSE NUMBER	TREATED MICE WEEKS 1		TREATED MICE WEEKS 2		CONTROL MICE WEEKS 1		CONTROL MICE WEEKS 2	
1		19.0* 15.0**		16.0		18.5 14.5		11.0
2		18.5 12.5		10.5		19.5 20.0		13.5
3		19.0 17.0		18.0		19.5 15.5		10.5
4		19.0 18.5		18.0		18.0 13.5		6.0
5		19.0 15.0		17.0		20.0 16.5		12.0

* WEIGHT OF ANIMAL AT START OF THERAPY.
** WEIGHT OF ANIMAL AT TIME OF TUMOR MEASUREMENT.

FIG. 3.

4-fold range. These results were based on data obtained in numerous experiments at the end of the course of injections. Mice in these experiments were subsequently observed for a prolonged period in order to note the appearance of delayed toxicity at the various dose levels employed. The maximum doses which cause a considerable degree of retardation of tumor growth, but no deaths due to acute or delayed toxicity are 5.0 mg/kg/day for SK 3818, 7.5 mg/kg/day for SK 4614, as compared to 0.25 mg/kg/day for SK 1133.

2. Picture of peripheral blood. Groups of 8 mice (RF Female, 20 ± 2 g) were studied during the course of injections with SK 3818 and SK 4614 (10 mg/kg/day for both compounds) 3, 5 and 7 days after beginning of therapy, corresponding to 7, 9, and 11 days after intramuscular implantation of Sarcoma 180. The red blood cell count was not altered during the course of injections. The white blood cell count of treated mice with and without tumors was characterized by a progressively increasing leucopenia as described for SK 1133(13), whereas the tumor bearing control mice showed a progressively increasing leucocytosis. The differential count varied from the one observed with SK 1133 (lymphopenia with relative increase of polymorphonuclear leucocytes) inasmuch as the granulocytes practically vanished from the picture of the peripheral blood toward the end of therapy, whereas the mononuclear cells showed a relative increase. Table III presents data obtained in treated tumor bearing mice and in the various control groups at the end of the course of the 7 injections of SK 3818 and SK 4614. Blood for counts was always collected from the base of the tail. In applying this method, the values obtained for the total white blood cell count were slightly lower in comparison to values calculated when blood was drawn from the end of the tail. The ratio as calculated from data

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13. Buckley, S. M., Stock, C. C., Crossley, M. L., and Rhoads, C. P., *Cancer*, in press, 1951.

TABLE III. Total and Differential Count of White Blood Cells in Mice Treated with SK 3818 and SK 4614.

Groups	Treatment, SK No.	Dose, mg/kg/day for 7 days		WBC, 1 mm^3 × 10^{-3}	PMN, %	MNC, %
I Normal mice without tumors	Phys. saline	—	Mean	10.2 (32)	18.5 (32)	81.5 (32)
			Range	6.2–14.2	11 –29	71– 89
II Control mice with tumors	Phys. saline	—	Mean	15.3 (12)	41.5 (12)	58.5 (12)
			Range	13.4–18	28.5–54	47– 71.5
III Treated mice without tumors	3818	10	Mean	1.3 (8)	1 (8)	99 (8)
			Range	.8– 2.4	0 – 2	98–100
IV Treated mice with tumors	3818	10	Mean	1.5 (8)	1 (8)	99 (8)
			Range	.6– 2	0 – 3	97–100
V Treated mice without tumors	4614	10	Mean	1 (8)	12 (8)	88 (8)
			Range	.8– 4.1	6 –16	84– 94
VI Treated mice with tumors	4614	10	Mean	1.9 (8)	2 (8)	98 (8)
			Range	1.1– 3	1 – 4	96– 99

WBC = White blood cell count.
PMN = Polymorphonuclear granulocytes.
MNC = Mononuclear cells, *i.e.*, lympho- and monocytes.
No. in parentheses = No. of mice.

obtained from groups of 8 normal mice (RF Female, 20 ± 2 g) is approximately 2 to 3.

3. Pathology.[†] (a) Administration of SK 3818 (10 mg/kg/day). Mice were immediately sacrificed and carefully autopsied 3, 5 and 7 days after beginning of therapy. During the course of injections, a considerable loss of weight occurred in both treated groups and tumor bearing control group. Diarrhea was not observed at any time in the treated mice.

Sarcoma 180. The tumors were in all instances smaller than the control tumors, especially in the groups receiving 5 and 7 injections. Histopathologically, the changes seen corresponded to the ones described for tumors of mice treated with 2,4,6-tris-(ethylenimino)-s-triazine(13) which were vacuolar degeneration of the cytoplasm, enlargement of nuclei and nucleoli, karyolysis, increased occurrence of karyorrhexis and pyknosis. The number of mitoses was decreased. All these changes were observed to some degree as early as 3 days after beginning of therapy.

Myeloid system and lymphoid system. An attempt was made to correlate the hematological data with the histopathological lesions seen in sternal and femoral marrow, spleen, thymus and lymphoid tissue of axillary and mesenteric lymph nodes and intestinal follicles. As early as 3 days after beginning of therapy, sternal and femoral marrow showed a considerable depletion. The bone marrow elements had disappeared from the sinusoids of the spleen. At the same time, the Malpighian bodies were not reduced in number, only the number of cells present was reduced. At the end of the experiment, the femoral marrow was fluid and purple red in all instances as compared to the pink and gelatinous marrow of untreated control mice. Sections revealed a severe depletion. In the spleen the myeloid elements were absent. A definite attack on the lymphoid elements was seen. Large forms of lymphocytes had disappeared during treatment. The small lymphocytes were decreased in thymus (especially in the cortical part), lymph nodes and spleen. Germinal centers were not observed. Only the intestinal follicles were relatively unaffected even after 7 injections. In summary then, a definite depletion of both systems was present at the end of therapy as reflected in the low total white blood cell count. However, SK 3818 appeared to damage the myeloid system more selectively at the dose level employed, a finding which was paralleled in the peripheral blood by granulocytopenia and relative increase of mononuclear cells.

Intestinal lesions. Parts of the gastro-intestinal tract (stomach, duodenum, jejunum, ileum, colon ascendens, colon transversum and rectum) were carefully searched for lesions after 3, 5 and 7 injections of SK 3818. There was shortening of the villi after 7 injections. Focal areas of edema, ulceration with inflammatory reaction and occasional hemorrhages were seen in the large intestine only. The most consistent lesions were located in the glandulae intestinales(14), where cells were necrotic at the base and in the middle part of the crypts. Pathological and incomplete mitosis was observed especially in the small intestine.

[†] We are indebted to Dr. John J. Buckley, Division of Laboratories and Research, New York State Department of Health, Sloan-Kettering Institute, for the histopathological examination.

Other lesions. Occasionally, there was vacuolar degeneration of tubules in both kidneys. In a few instances, the livers showed areas of necrosis and hemorrhage after seven injections of the compound; lung, heart, voluntary muscle, pancreas and both adrenals were free of lesions.

There was no difference between the findings of treated mice with tumors and treated, non-tumor bearing animals. (b) Administration of SK 4614 (10 mg/kg/day). The experimental procedures were the same as employed for the investigation of SK 3818. The histopathological changes were essentially the same as described for SK 3818, only to a lesser degree (intestine, kidney, liver). There was essentially no difference between the findings of treated mice with tumors and treated, non-tumor bearing animals.

Discussion. SK 3818 and SK 4614, two N-ethylene substituted phosphoramides, by an injection course of 7 days, were able to retard the growth of Sarcoma 180 whether started 24 hours or 4 days after implantation of tumor grafts. These results present new evidence for the observation that anti-tumor activity is associated with the presence of ethylenimine groups. The two phosphoramides are related in their reactivity to the ethylenimonium intermediates occurring during the hydrolysis of the nitrogen mustards. It was not surprising therefore to find lesions similar to the ones induced by nitrogen mustards(15), ethylenimino-s-triazine and some ethylenimine derivatives of urea(5,13,16), namely, histopathological manifestations limited to tissues characterized by a high degree of proliferation such as the hematopoietic system, the intestinal mucosa and various experimental tumors(17,18). In their greater adverse effect against the myeloid system, SK 3818 and SK 4614 resemble the ethyleneureas(13). It remains to be investigated if any clinical value will be attributed to this observation, *i.e.,* selective administration of the two compounds for cases of myelogenous leukemias rather than of lymphatic leukemias. However, it is scientifically and especially chemotherapeutically of interest to note that by changing the structure of the nucleus that contains ethylenimine groups the cytotoxic action can be varied somewhat. The lesions in the intestine correspond to the mode of action of nitrogen mustards on intestinal epithelium of the rat as studied recently(14).

Perhaps of practical value is the observation that SK 3818 has shown a greater range of effective dose levels than those observed with SK 1133, 2,4,6-tris(ethylenimino)-s-triazine and other ethylenimines that have been tested. For this reason SK 3818 might prove better than SK 1133 for maintenance therapy. Clinical trials of the phosphoramides are in progress. The results of those studies(19) will throw light on any possible clinical advantages of N,N′,N″-triethylenephosphoramide over 2,4,6-tris(ethylenimino)-s-triazine.

Summary. 1. SK 3818, N,N′,N″-triethylenephosphoramide and SK 4614, N,N-diethyl-N′, N″-diethylenephosphoramide are both capable of retarding the growth of Sarcoma 180 in mice 24 hours and four days after implantation of tumor. Among the chemotherapeutic agents tested in our laboratory SK 3818 has shown the greatest effective dose range. 2. Histopathological examination of tumors of mice treated with SK 3818 and SK 4614

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showed enlargement of cells with vacuolar degeneration of cytoplasm, enlargement of nuclei and nucleoli. Furthermore, the number of mitoses was decreased. Numerous cells showed nuclear alterations as commonly encountered during necrosis such as pyknosis, karyolysis and karyorrhexis. 3. Histopathological examination of organs of mice treated with SK 3818 and SK 4614 revealed lesions similar to the ones occurring during administration of nitrogen mustards: intestinal damage and depletion of myeloid and lymphoid system with leucopenia (granulocytopenia and relative increase of mononuclear cells) in the peripheral blood.

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The Central Nervous Depressive Effect of Khellin. (19055)

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Khellin, a glucoside isolated from the plant *Ammi visnaga*(1-3) has been tested clinically in recent years as an antispasmodic agent and a coronary dilator, principally in the management of angina pectoris. Clinical results from different laboratories are varied both as to its efficacy as a smooth muscle relaxant and its toxic effects(4-8). Insomnia, somnolence, light-headedness, dizziness, nausea and vomiting have occurred in patients receiving therapeutic doses of the drug. While the pharmacological action of Khellin on the cardiovascular system and the smooth musculature has been extensively investigated (9-11), no study apparently has been made of its central nervous action. In our toxicological studies, it was observed in mice that Khellin both depresses and excites the central nervous system. Depression occurs prior to excitement, which appears only with large doses. The depression is mild but of long duration. Excitement begins with an increase in motor activity, followed by clonic convulsions indicating stimulation of nervous centers above the medullary levels. Such an action may produce some of the toxic effects of Khellin in man.

Since sedation is of value therapeutically in patients suffering from angina pectoris or from chronic bronchial asthma(12,13), the central depressive action of Khellin may be likewise effective in the relief of angina or asthmatic attacks. With such a view in mind, the central nervous depressive action of Khellin was investigated in cats, rats and mice. The results are presented in this report.

Methods and results. A. *Anti-caffeine effect.* This was determined with jiggle cages in rats receiving a stimulating dose of caffeine by the procedure of Schulte and associates(14). Activity was measured and recorded in terms of the total number of revolutions of the work

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TABLE I. Effect of Khellin on Activity of Rats Stimulated by Caffeine.

Caffeine, mg/kg S.C.	Khellin		Work performance		
	mg/kg oral	Toxic signs	Revolutions, 5 hr	Diff. in activity	% reduction of activity*
0	0		25.4		
30	0		97.1	61.7	
30	2.5		69.2	43.8	29
30	10		61.2	35.8	42
30	20		38	12.6	79.6
30	40	Slight depression	23.3	–1.9	100

$$* \frac{\text{Activity by caffeine alone} - \text{activity by caffeine plus khellin}}{\text{Activity by caffeine alone}} \times 100.$$

TABLE II. The Anticonvulsant Property of Khellin.

Anti-electroshock activity							Antimetrazol activity		
Cats			Rats		Mice		Rats		
Dose, mg/kg oral	Toxic signs	Rating	PD_{50}, mg/kg oral	Toxic signs	PD_{50}, mg/kg oral	Toxic signs	Dose, mg/kg oral	Toxic signs	Rating
200* single	Mod. dep.	1+	42	Sl. dep. at 30	250	Sl. dep. at 25	500§	Sl. dep.	4+
100† 2 daily	Deep dep.	3–4+					250	”	4+
							125	”	3+
50† 2 daily	Sl. dep.	0–1+					63	”	1+
							31	None	0

* 3 cats, one died next day.
† 3 ” all ” ” ”.
§ Lethal.

TABLE III. Combined Hypnotic Effect of Khellin and Pentobarbital Sodium in Mice.

No. animals used	Khellin, mg/kg oral	Pentobarbital-Na, mg/kg Ip.	Sleeping time		"P"
			Mean ± S.E. (min)	Diff.*	
40	0 (control)	50	23.44 ± 1.04		
40	1	50	37.15 ± 1.04	13.71	.01
20	2.5	50	50.12 ± 1.09	26.68	.01
20	5	50	91.20 ± 1.15	67.76	.01

* Difference in avg sleeping time between controls receiving pentobarbital only and mice receiving pentobarbital plus khellin.

adder throughout the experiment of 5 hours. Caffeine alkaloid, 30 mg/kg in 5 ml of 0.9% saline, was given subcutaneously; within 2 minutes, Khellin suspended in 7% gum acacia was administered orally. Fifteen male Sprague-Dawley rats weighing around 150 g, without food for 16 hours, were used for each dose level. The results in average values are given in Table I. It is seen that 10 mg/kg of Khellin, a non-hypnotic dose, will suppress about half the activity induced in rats with 30 mg/kg of caffeine.

B. *Anticonvulsant property*. The anticonvulsant activity of Khellin was investigated against electro-shock convulsions in cats by the method of Putnam and Merritt(15) and in rats and mice by the method of Toman, Swinyard, and Goodman(16), and against metrazol-induced convulsions in rats by a procedure described elsewhere(17). As the results in Table II indicate, the anticonvulsant activity of Khellin is attributable to its sedative and hypnotic effect. Khellin is more toxic for the cat than for the rat or the mouse. No excitement occurred in the cat after ingestion of a lethal dose of Khellin.

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C. *Hypnotic effect.* Hypnosis does not occur in animals receiving increasing dosage of Khellin. To study the influence of Khellin on hypnosis, the joint effect of Khellin and pentobarbital was determined in mice by the duration of sleep. The increase in sleeping time due to Khellin was taken as a measure of its central depressive effect. The results in Table III were obtained in groups of 20 and 40 mice which received a suspension of Khellin in 7% gum acacia orally half an hour before 50 mg/kg of pentobarbital was injected intraperitoneally. The duration of pentobarbital-hypnosis was definitely prolonged with 1 mg/kg of Khellin; with 5 mg per kg it was 4 times as long as that by pentobarbital alone.

Summary. Khellin has been shown to be effective in suppressing excitement in rats induced by caffeine. At slightly depressive levels, it is more effective against metrazol than against electrically induced convulsions. The central nervous depression produced by Khellin is mild but of long duration; with increasing dosage this is followed in mice by excitement.

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Factors Contributing to the Difference in Propylthiouracil Goitrogenesis in Rats and Guinea Pigs. (19056)

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In marked distinction to the rat, dietary thiouracil goitrogenesis in the guinea pig requires prolonged administration of the drug (1,2). On the other hand, administration of thyroid stimulating hormone readily produces a goiter(3). It seems probable that the explanation of this species difference would be concerned with one or all of the following classes of factors: (1) the concentration and effectiveness of thiouracil in the thyroid; (2) the rates of synthesis, excretion and utilization of the thyroid hormone; and (3) the threshold for increased thyrotrophin production and the goitrogenic capacity.

This report is concerned with two questions: (1) Is propylthiouracil equally effective in inhibiting thyroid hormone synthesis in both the rat and the guinea pig, and (2) Is thyroid hormone synthesized at comparable rates in these species. We have investigated these problems by measuring the effect of propylthiouracil on the rate of I^{131} incorporation into the protein of rat and guinea pig thyroid slices *in vitro*. That I^{131} incorporation under these conditions represents thyroid hormone synthesis has been shown by the work of Morton and Chaikoff(4).

Methods. Guinea pigs weighing 600-800 g, and Sprague-Dawley rats weighing 150-250 g were used regardless of sex. They had been fed stock diet for at least 2 weeks prior to use. The animals were sacrificed by a blow on the head, and the thyroids were rapidly removed, trimmed, weighed and transferred to iced containers. The glands were sliced as uniformly as possible. This usually meant division of each lobe into 3 to 4 slices for the guinea pig, and into 2 for the rat. When necessary, the lobes from several animals were combined to furnish sufficient tissue for the separation procedures outlined below. All glands were divided at the isthmus, and slices from one lobe were incubated with propylthiouracil, while those from the other lobe were used in the control flasks. The thyroid slices were incubated for 3 hours at 38°C in Krebs-Ringer's solution which contained 10-30 μc of I^{131} per ml. In the flasks containing propylthiouracil, concentrations ranging from 10^{-2} M through 10^{-7} M were used. At the end of the experiment the reaction was stopped by boiling, and the tissue was homogenized in an all-glass instrument. The protein was precipitated with 20% trichloroacetic acid, kept at room temperature for one hour, and washed twice in 5% trichloroacetic acid. The precipitated protein was dissolved by heating in 4-10 ml of 4N NaOH for 6 to 12 hours, and appropriate aliquots evaporated on metal planchets without adjustment of pH. The radioactivity in these preparations was estimated with an end-window Geiger-Mueller tube and compared with standards appropriately prepared to permit corrections for absorption and decay. The results were calculated as:

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$$\frac{\%\text{ of dose bound to protein}}{\text{mg thyroid tissue}}$$

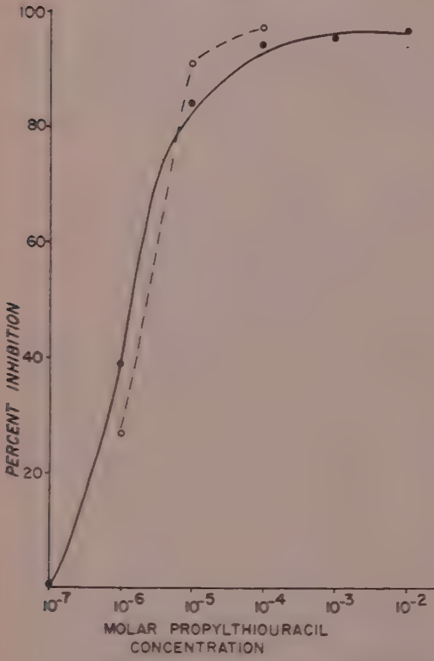

FIG. 1. The relation between propylthiouracil concentration and I^{131} binding to thyroid protein *in vitro*. Solid line, guinea pig thyroid; broken line, rat thyroid.

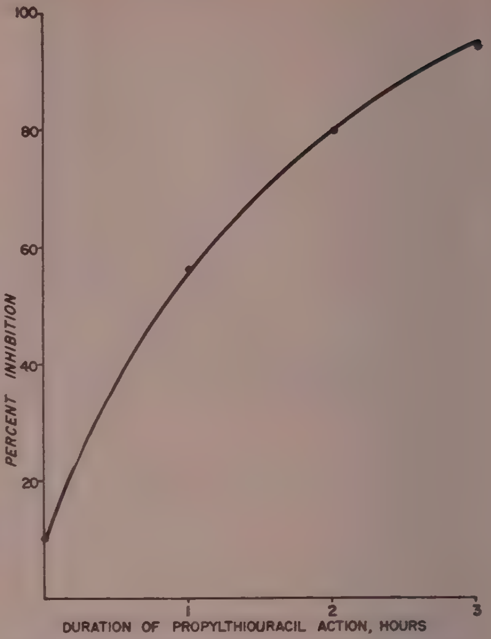

FIG. 2. The relation between duration of propylthiouracil action and I^{131} binding to guinea pig thyroid protein.

Results. In Fig. 1 the relationship between protein binding of I^{131} by surviving rat and guinea pig thyroid slices and the concentration of propylthiouracil in the incubation medium is shown. In both cases thyroid response was similar. At a concentration of 10^{-7} M, propylthiouracil was without effect; 10^{-6} M caused 25-40% inhibition; and 10^{-5} M resulted in almost complete inhibition of I^{131} uptake. The reality of these effects was tested by adjusting the propylthiouracil concentration in experimental flasks to 10^{-5} M at various intervals after incubation of guinea pig thyroid slices with I^{131} (Fig. II). When propylthiouracil was added at zero time, little or no I^{131} was incorporated into gland protein; and when added one or 2 hours after the addition of I^{131}, incorporation was reduced to approximately 80 and 60% of the 3-hour values, respectively. Consequently it appears that propylthiouracil *in vitro* inhibits thyroid hormone synthesis in guinea pig thyroid, and that the effectiveness of this drug in the guinea pig is not materially different from its effectiveness in the rat.

TABLE I. Relative Incorporation of I^{131} into Protein of Rat or Guinea Pig Thyroid Slices *in vitro*.

	No. of exp.	Avg. % of dose bound to protein/ mg of thyroid tissue	Stand. error
Rat	7	.494	.069
Guinea Pig	14	.0930	.0099

Table I presents data concerned with the relative activity of thyroid slices from both species with respect to I^{131} binding. It can be seen that rat thyroid slices incorporated I^{131} into the protein of the gland about five times as rapidly as did guinea pig thyroid slices. In the light of Morton and Chaikoff's work (4), it would appear that the rate of thyroid hormone synthesis is very much greater in the rat than in the guinea pig.

Discussion. The observations reported above show that, *in vitro*, propylthiouracil is equally effective in inhibiting thyroid activity in both the rat and the guinea pig. It is reasonable, therefore, to expect the same phenomenon to occur *in vivo* provided the drug reaches the acinar cells in adequate concentration. Recalculation of the data of Williams and coworkers(3,5) indicates that oral administration of the drug results in gland concentrations several hundred fold greater than that necessary to produce complete inhibition of iodine binding *in vitro*. These values were achieved within a very few days after the drug was given. Therefore, it hardly seems reasonable that the species difference in thiouracil goitrogenesis results from differences in the availability of the drug.

A more attractive hypothesis accounting for the refractoriness of the guinea pig to propylthiouracil is concerned with the balance between hormone production and its peripheral utilization. In the absence of thyroid activity, the duration of the euthyroid state would be inversely proportional to the rate of peripheral utilization of the hormone. Consequently the time lag before stimulation of pituitary thyrotrophin production and the subsequent appearance of the goiter should be proportional to the rate at which thyroid hormone was utilized.

Our observations, admittedly far from conclusive, provide evidence consistent with this hypothesis. It is obvious that in the intact euthyroid animal the rates of thyroid hormone production and utilization must be balanced. Measurement of the rate of hormone production should then shed light on the rate of utilization. Our data indicate that hormone synthesis by the rat thyroid occurs at approximately 5 times the rate for the guinea pig thyroid. Other factors remaining equal, it follows that peripheral hormone utilization and equivalent levels of hypothyroidism would occur in the guinea pig at one-fifth the rate for the rat. Thus the time lag for propylthiouracil goitrogenesis should be 5 times as long, about 50 to 70 days. This estimate agrees well with the observations reported by D'Angelo(1) and MacNair and Young(2).

Summary and conclusions. (1) The difference between the rat and guinea pig with respect to thiouracil goitrogenesis was investigated using the binding of I^{131} to surviving thyroid slices as a measure of thyroid hormone production. (2) Propylthiouracil in concentrations ranging from 10^{-7} M through 10^{-5} M inhibited hormone synthesis to an equal extent in both rat and guinea pig thyroid slices. *In vivo* studies have indicated the compound to concentrate in the gland to a comparable degree in both species. On the other hand, in the absence of propylthiouracil, hormone synthesis in rat thyroid slices occurred at 5 times the rate for guinea pig thyroid slices. (3) In view of these observations it is suggested that the species difference in thiouracil goitrogenesis can be accounted for by differences in the rates of peripheral utilization of circulating thyroid hormone.

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Alkaline Phosphatase—Correlation of Histochemical Demonstrability in Pancreatic Tissue with Presence in Pancreatic Juice. (19057)

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Gomori(1) and subsequently others(2-4), have reported that the histochemical method for demonstration of alkaline phosphatase produces a strongly positive reaction in the centro-acinar and intralobular duct cells of the dog. It has also been shown that pure pancreatic juice from the dog contains alkaline phosphatase(4,11).

In the present studies observations on the histochemical reaction for alkaline phosphatase have been extended to the pancreas of other species and samples of the pancreatic juice of these same species have been examined for their content of alkaline phosphatase.

Pancreatic tissue and pancreatic juice from man, dog, cat, guinea pig and rabbit were studied. The histochemical demonstration of alkaline phosphatase was accomplished by Gomori's method(5) after fixation of the tissue in 80% ethyl alcohol. Alkaline phosphatase in the pancreatic juice was determined by the Shinowara modification(6) of the Bodansky method with color development according to the procedure of Holman(4). In the animals, pancreatic juice was obtained by direct cannulation of the major duct and stimulation with purified secretin.* Pancreatic juice was obtained from 2 human patients with pancreatic fistulas. In each case it was demonstrated during subsequent surgery that the pancreatic fistula did not communicate with the duodenum. Thus, in neither the animal nor the human experiments was there contamination with duodenal juice, which is known to contain alkaline phosphatase(7). The animal tissues were removed before death and immediately placed in the fixative. The human pancreatic tissue was obtained by surgical biopsies.

The findings were as follows (Table I). The intralobular ducts and centroacinar cells of the dog, rabbit and human showed a positive staining reaction for alkaline phosphatase and the pancreatic juice of these species contained the enzyme. In the guinea pig and cat the enzyme was not demonstrable in either the tissue or the juice. The acinar cells of all the species examined were negative for alkaline phosphatase.

Comment. In an earlier study we have shown that elevation of the level of alkaline phosphatase in the blood does not cause an increase in the amount in the pancreatic juice (8). Taken together with the present evidence, this indicates that the alkaline phosphatase of the pancreatic juice is supplied by the pancreas itself and that, at least for this component, the intralobular ducts and centro-acinar cells have a secretory function. The evidence supporting the possibility that the intralobular ducts and centro-acinar cells have other secretory functions has been reviewed elsewhere(9). In the cat alkaline phosphatase is demonstrable in the walls of the blood vessels after freezing-drying fixation of the pancreas, but the intralobular ducts and centro-acinar cells are negative just as they are with alcohol fixation(10).

Summary. In man, dog and rabbit alkaline

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* Kindly supplied by Dr. E. D. Campbell of the Lilly Research Laboratories.

TABLE I. Alkaline Phosphatase in Pancreatic Tissue and Pancreatic Juice of Various Species.

		Pancreatic juice	
Species	Reaction of intralobular duct to stain for alkaline phosphatase	No. of specimens	Alkaline phosphatase (units/100 cc)
Dog	+	124	.4 to 41
Human	+	2	2.1 to 3.8
Rabbit	+	8	2.6 to 4.4
Guinea pig	0	3	0
Cat	0	10	0

phosphatase is demonstrable histochemically in the intralobular ducts and centro-acinar cells of the pancreatic tissue and is present in the pancreatic juice. In the cat and guinea pig this enzyme is present neither in the intralobular ducts and centro-acinar cells nor in the pancreatic juice.

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Development of Resistance to 4-Amino-N^{10}-Methyl Pteroylglutamic Acid (Amethopterin) by *Streptococcus faecalis*.* (19058)

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In children with acute leukemia, whose disease initially responds well to treatment with 4-amino-N^{10}-methyl pteroylglutamic acid (amethopterin), there develops in the leukemic process, after 6 to 18 months, refractoriness to therapy(1-4). Prevention of this development of resistance would be of the greatest clinical importance, since in the absence of resistance these children could be treated again and again and the disease suppressed indefinitely. The development of resistance to amethopterin in a previously sensitive strain of mouse leukemia has been reported by us(5), and was later repeated in another strain of leukemia by Law and coworkers(6). With these two strains of amethopterin-fast leukemias, studies are in progress on the mechanisms of resistance in the hopes of finding technics for preventing its development. In order to provide another system for the study of resistance to amethopterin, attempts were made to develop an amethopterin-fast strain of *Streptococcus faecalis*, an organism ordinarily requiring folic acid, and very sensitive to the inhibitory action of amethopterin.

Method. Streptococcus faecalis (ATCC No. 8043) was grown on Difco folic acid assay broth in the presence of 5 mγ/ml of pteroyl-

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† Senior Fellow of the Damon Runyon Memorial Fund for Cancer Research.

‡ Research Fellow of the National Cancer Institute.

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glutamic acid (PGA), and then inoculated into similar broth containing the same amount of PGA, and concentrations of amethopterin varying from 1 to 128 mγ/ml. In 24 hours there was visible growth only in the tubes containing 2 and 4 mγ/ml, but after 48 hours there was slight growth in the tube containing 32 mγ/ml. No growth occurred, however, after 5 days in the tubes containing 64 or 128 mγ/ml. The original strain was then inoculated into 4 pour plates containing 5 mγ/ml of PGA and 0, 10, 20, and 80 mγ/ml respectively of amethopterin. In 24 hours, there was heavy growth only in the plate containing no amethopterin, but after 96 hours one colony each appeared on the 10 and 20 mγ/ml plates, but none on the 80 mγ/ml plate. The colony from the 20 mγ/ml plate was inoculated into broth containing PGA and various concentrations of amethopterin. Twenty-four hours later, good growth was seen at 8, 16, and 32, but not at 64 mγ/ml. An inoculum from the highest concentration allowing growth was then transferred into pour plates containing increasing amounts of amethopterin. Colonies were picked after 72 to 120 hours from the plate containing the highest concentration of antimetabolite and inoculated into amethopterin containing liquid media. This alternation between liquid and solid media was continued for 16 transfers, after which time only liquid media was used. Media used in the first 6 transfers contained 5 mγ/ml of PGA. After that time 10 mγ/ml was used.

Studies on the ability of the resistant strain to utilize certain antimetabolites were done in Difco folic acid assay liquid media without the addition of PGA.

Results. As can be seen from Fig. 1, resistance to amethopterin appeared gradually in a stepwise fashion. On the 6th transfer, a few colonies grew on the plate containing 5 mγ/ml PGA, and 1,000 mγ/ml of amethopterin. By the 14th transfer, a few colonies grew out in the plate containing 20,000 mγ/ml. After a total of 28 transfers, good growth occurred in 20-24 hours in liquid media containing 10 mγ/ml PGA, and 40,000 mγ/ml amethopterin. It is now being maintained in media containing 50 γ/ml amethopterin.

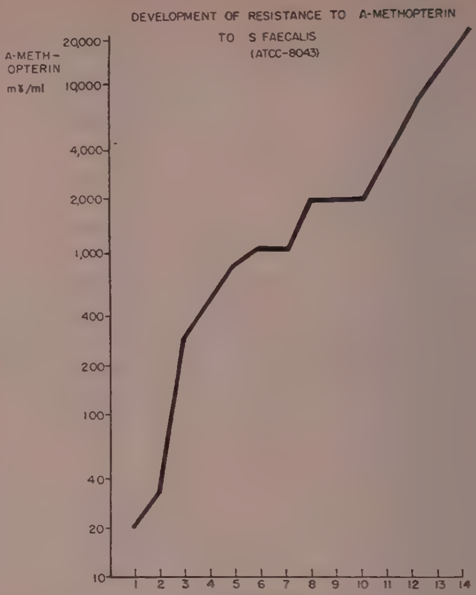

FIG. 1.

No morphologic differences between the sensitive and resistant variants could be detected in Gram stained preparations. The resistant organism had a smaller quantitative requirement of PGA (0.1 mγ/ml) for half maximum growth in the absence of amethopterin than did the parent sensitive strain (0.3-0.5 mγ/ml). In the presence of 1 to 25,000 mγ/ml of amethopterin, however, the resistant strain grew without PGA. 4-amino-PGA (aminopterin) and 4-amino-pteroylaspartic acid (amino-an-fol) could also be utilized by this organism to supply its PGA requirements.

Discussion. The development of resistance to amethopterin by *Streptococcus faecalis* is more analogous to the resistance to penicillin (7,8), chloromycetin(9), aureomycin(8,10),

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7. Demerec, M., *Proc. Nat. Acad. Sci.*, 1945, v31, 16.

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10. Gezon, H. M., Fasan, D. M., PROC. SOC. EXP BIOL. AND MED., 1950, v73, 10.

and terramycin(11-13), than to streptomycin (14), in that it occurs gradually in a stepwise fashion, and in that there has been so far no demonstration of an amethopterin-dependent strain of this organism.

This resistance is presumed to be due to a random mutation selected out by the presence of the drug as previously demonstrated for *Staphylococcus aureus* with both penicillin and streptomycin(7,14).

The fact that the resistant bacterial cell is able to utilize 4-amino PGA and 4-aminopteroyl aspartic acid, as well as amethopterin, to supply its PGA requirement is of considerable interest. The mechanisms by which this resistance and this ability to utilize amethopterin are achieved, are under study at the present time.

Summary. (1) By growing *Streptococcus faecalis* (ATCC No. 8043) in a medium containing successively higher concentrations of amethopterin, more than a thousand-fold increase in resistance has been achieved. (2) This amethopterin-fast variant has a slightly lower requirement for folic acid in the absence of amethopterin than the parent sensitive strain. In the absence of folic acid, however, amethopterin and certain other 4-amino derivatives of PGA can replace this folic acid requirement in the resistant strain.

We are greatly indebted to Dr. H. Christine Reilly for her suggestions and advice in these studies.

Received September 24, 1951. P.S.E.B.M., 1951, v78.

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14. Demerec, M., *J. Bact.*, 1948, v56, 63.

Evaluation of Some Iodine-Containing Organic Compounds as X-Ray Contrast Media. (19059)

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Organic compounds containing iodine have been useful aids in radiographic diagnoses. We have previously studied the physiological behavior of one of these, Urokon(1), which has proven of value in the visualization of the pelves and ureters of the kidneys of man(2). Five analogues of Urokon and a number of other iodine-containing compounds have now been investigated. Some of these which are of experimental and potential clinical interest are listed in Table I. Two approaches to the determination of their value have been made, radiographic study of the compounds after intravenous or oral administration (dogs) and analyses of the iodine content of kidney, liver, and blood after intravenous administration (rats).

Roentgenographic studies. Dogs 5 to 9 kg in weight were maintained in the laboratory over several months, so that the same animals might be used in a comparative series of investigations with various potential cholecystographic agents. A portable Picker x-ray unit was employed with instrumental settings at 50 kilovolts and 84 milliamperes and exposures of 1 to 1.75 sec, depending on the size of the animal. Roentgenograms were taken at intervals as shown in Table II. Under these conditions, it was possible to obtain clear visualization of the gall bladder and biliary ducts. For intravenous injection, 10% solutions (pH 7-9) of the sodium salts (sodium carbonate as neutralizing agent) of the compounds studied were used. After a 20 hr fast, the dog was fed the yolk of a hard boiled egg, 4 hr preceding the injection of

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1. Neuhaus, D. R., Christman, A. A. and Lewis, H. B., *J. Lab. Clin. Med.*, 1950, v35, 43.

2. Nesbit, R. M., and Lapides, J., *J. Urol.*, 1950, v63, 1109.

TABLE I. Compounds studied.

Chemical name	Identification No.	LD_{50} in mice mg/kg	Iodine %
3-Formylamino-2,4,6-triiodobenzoic acid	1	8000	70.0
3-Acetylamino-2,4,6-triiodobenzoic acid*	2	8000+	68.4
3-Propionylamino-2,4,6-triiodobenzoic acid	3	8100	66.7
3-Butyrylamino-2,4,6-triiodobenzoic acid	4	—	65.1
3-Caproylamino-2,4,6-triiodobenzoic acid	5	1450	62.1
N-(3-amino-2,4,6-triiodobenzoyl)-ε-amino-n-caproic acid	6	410	61.7
2-(4-hydroxy-3,5-diiodobenzyl) benzoic acid	S	—	52.9
α-Phenyl-β-(4-hydroxy-3,5-diiodophenyl) propionic acid†	P	—	51.5

* This compound, as the sodium salt, is known as Urokon Sodium.
† Priodax.

the compound. This preliminary treatment is necessary to obtain clear visualization of both the gall bladder and the biliary ducts after injection of the compounds. After the dog was anesthetized (35 mg of sodium pentobarbital per kg) and placed on its right side on the table, a scout film was made to establish the proper length of exposure and then the experimental solution was slowly injected intravenously over a period of 3-5 min. After repeated anesthesia and exposure to x-ray, the anesthetic was frequently effective for as long as 8 hr rather than 3 hr as in the initial experiments. Dogs D and E, which had been injected and x-rayed approximately 60 times over a period of 3 months, were sacrificed. Histological examination showed normal liver, sternum and ribs; the hematocrit values were slightly low however. Since these animals had been used more frequently than any of the others of the entire group, it is believed that the experiments, details of which are reported in Table II, were carried out on essentially normal animals.

The experimental procedure for the study of compounds after oral administration was similar to that already described for intravenous injection and to that used by Jones and coworkers(3). The compound to be studied was mixed with boiled egg yolk, crumbled and moistened to a suitable consistency with water. This mixture was fed 8 hr after food had been removed from the cage. After a further period of 16 hr, the animals were anesthetized and roentgenograms were taken as described. Occasionally an animal required additional pentobarbital to maintain satisfactory anesthesia for the 6 hr experimental period (*i.e.*, 16th to 22nd hr after feeding the egg yolk-compound mixture); in these cases, one fourth of the amount given intravenously was injected intramuscularly. No symptoms of toxicity (nausea, vomiting, diarrhea, etc.) were observed with any of the compounds listed in Table I. In the investigation of Jones and coworkers(3), the time required for the initial visualization of the biliary ducts and gall bladders after administration of their new compound, 2-(4-hydroxy-3,5-diiodobenzyl) benzoic acid (Compound S of Table I), was compared with that for Priodax, Isoiodeikon, and Iodeikon. Compound S showed a distinct advantage over the others. In Table II, Compound S and Priodax (P), given under our conditions, are compared with Urokon and its analogues (Compounds 1-5) and Compound 6. After intravenous injection differences are small; Compound S how-

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3. Jones, G. E., Grohowski, A. S., Robertson, H. D., Ramsey, G. H., Schilling, J. A., and Strain, W. H., *Radiology*, 1948, v51, 225.

TABLE II. Initial Visualization of Biliary Ducts and Gall Bladders of Dogs after Oral and Intravenous Administration of Iodine-Containing Compounds.

			Time interval of initial visualization compound administered							
Tissue visualized	Dosage	Dog No.	S	P	6	5	4	3	2	1
				Intravenous						
	mg/kg		min	min	min	min	min	min	min	min
Gall bladder	53	B			20					
	60	B	40							
	75	B	20	20	40	20	40	—	—	60*
	75	E	20	60	40					
			100		40					
	75	F	20	—	20	20	20	20	—	
Biliary ducts	53	B			20					
	60	B	20							
	75	B	20	40	40	20	40	—	—	60*
	75	E	20	40	40					
			20		40					
	75	F	20	—	20	20	20	20	—	
				Oral						
			hr	hr	hr	hr	hr	hr	hr	hr
Gall bladder	50	B	19		19		—		—	
			—		16					
	100	D	19		22					
	44	F			19					
	50	F	19		16	—		—		
Biliary ducts	50	B	16		16		16		22	
			16		16					
	100	D	16		16					
	44	F			16					
	50	F	16		16	16		19		

* Appeared only on one film.

ever is somewhat better than Compound 6 in visualization of the biliary ducts and inferior with respect to the gall bladder. After oral administration, they are of approximately equal value in both respects. On the other hand, visualization of the gall bladder was not obtained after oral administration of Urokon or any of its analogues (Compounds 1-5). Perhaps the reason for this can be found in the fact that this group included the only compounds tested which made possible the visualization of the kidneys within 3 hr after injection. Epstein and his associates(4) have noted that the more water-soluble compounds appeared rapidly in the urinary tract and were less likely to give clear biliary contrast on x-rays. Urokon (Compound 2) has been shown to be an excellent pyelographic medium (1).

In view of these results it is interesting to note from Table I that compounds 5 and 6 are very similar in structure. The prime difference lies in the placement of the caproyl group (Compound 5) and the polarity of Compound 6. The lack of similarity in their LD_{50} values in mice (Table I) and in their biological behavior in dogs (Table II) may be related to this difference in chemical structure.

Chemical analysis of tissues. The x-ray studies already described have demonstrated that the various compounds under discussion are concentrated in and excreted by the liver and kidneys at varying rates. Further important information should be obtained if the tissues were examined for their content of the radiographic dyes at varying intervals after administration. Rats of 100 to 150 g weight were fasted overnight and received intravenously through the tail vein the compound under investigation at a level of 50 mg per kg body weight; the sodium salts were prepared as in the previously described studies. The animals were chloroformed, blood samples

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4. Epstein, B. S., Cohen, H. L., and Naletson, S., *Radiology*, 1950, v54, 87.

were taken from the left ventricles immediately after death, and the liver and kidneys were removed for analysis. The analytical procedure was essentially that of Matthews, Curtis and Brode(5) for total iodine as modified in this laboratory(1). The minimal number of animals in each group was 3; if the results were widely variant, additional animals were used to establish a more accurate value. Results presented in Table III are the average of values obtained from at least 3 animals.

From an examination of Table III, it can be seen that Urokon and its analogues (Compounds 1 to 4), having from one to 4 carbons in the acyl group, were present in the kidneys in large amounts 10 min after injection; by 180 min, however, the concentration had fallen to a relatively low level. Although Compound 5 did not reach as high an initial level as the other analogues, its concentration in the kidney during the final intervals was comparable with those of Compounds 1 to 4. By way of contrast, the amounts of Compounds 6 and S during the first 20 min were lower than those of the Urokon series, but 60 to 180 min after injection the values were considerably higher. Concurrently, the levels of all of the Urokon analogues per ml of blood fell rapidly and to a relatively low concentration by the final analytical period whereas the corresponding values for 6 and 8 remained somewhat higher.

When the concentration of radiographic dye in the kidney tissue is plotted against that in blood for each interval, the resulting curves demonstrate the rate of excretion of the dyes with increasing amounts present in the blood. In Fig. 1 the results for 3 of the analogues are plotted; the curve for Compound 2 is very similar to those for 1 and 3, and was selected to represent the curves for all 3 compounds. From the figure it can be noted that with the decreasing length of the acyl group, the slopes of the curves are steeper. Thus the rate of excretion of Compound 5, is slower than that of either Compound 2 or 4. In the cases of Compounds 4 and 5, the rates of excretion appear to reach a maximum as the amounts of the respective compounds in the blood are increased, but for Compound 2, no plateau was obtained in the range studied.

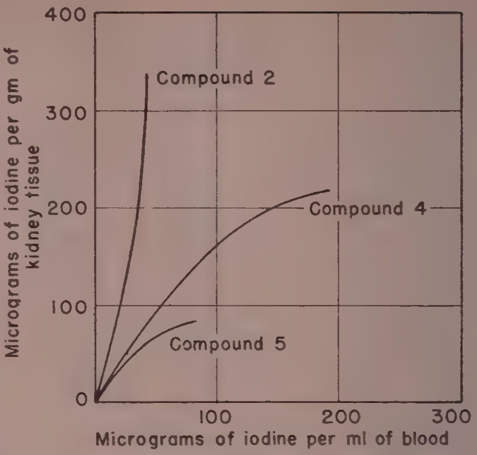

FIG. 1. Rates of Excretion of Compounds 2, 4 and 5.

The results of the analyses of the liver after intravenous injection of these compounds (Table III) indicate that those which are excreted slowly by the kidneys are found in comparatively greater amounts in the liver. After 10 min the concentrations of Compounds 1 to 5 and S are not widely different, but after 60 min, the concentrations of the group 1, 2 and 3 are significantly lower than those of the other compounds. In the final analytical period, the concentrations of Compounds 1 to 3 are at a relatively low level. Of all of the compounds tested, it is only Compound 6 that is consistently in greater concentration throughout the three hour period. This evidence corroborates the roentgenographic findings described above; after intravenous injection the four compounds, 4, 5, 6 and S, which were maintained at a higher concentration in the livers of rats than Compounds 1 to 3, were those which were effective cholecystographic agents in dogs.

From the results of the experiments on rats, it can be concluded that Compounds 1 to 3 are too rapidly excreted by the kidneys to be of probable value as cholecystographic agents after either oral or intravenous administration. This is borne out by the roent-

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5. Mathews, N. L., Curtis, G. M., and Brode, W. R., *Indust. Eng. Chem.* (Anal. Ed.), 1938, v10, 612.

TABLE III. Concentration of Iodine in Blood, Kidneys, and Liver of Rats after Intravenous Injection of Cholecystographic Media.

All values are expressed μg of iodine per g or ml

Tissue analyzed	Interval after inj.	Compound						
		1	2*	3	4	5	6	S
Kidney	10	263.	363.	278.	217.	80.3	66.5	56.5
	20	125.	199.	105.	128.	111.7	78.	76
	30	120.	120.	136.	86.4	73.8	94.9	64.9
	60	31.	48.	27.5	54.	38.8	82.5	45.9
	120	10.4	6.6	20.9	22.7	22.	79.5	35.4
	180	8.6	9.2	3.7	12.7	9.4	55.1	23.7
Blood	10	50.1	40.5	54.	194.	83.5	22.7	76.9
	20	33.1	31.2	28.	55.9	45.4	57.3	66.6
	30	24.2	26.5	18.8	45.	38.9	45.6	62.4
	60	6.	6.6	8.	31.1	26.1	50.5	44.4
	120	1.	3.6	3.6	16.6	10.2	26.7	26.5
	180	0.	0.7	1.3	9.8	6.	14.8	23.4
Liver	10	45.6	48.8	73.6	73.7	68.	125.	59.7
	20	22.8	42.8	48.1	47.3	64.5	137.	60.2
	30	14.1	38.2	30.7	42.3	64.8	129.	48.3
	60	6.4	11.4	19.9	31.8	52.9	130.	28.2
	120	.6	1.6	4.8	22.	40.4	108.	17.6
	180	.0	.0	5.3	13.3	20.9	70.8	15.

* Urokon.

genographic studies on dogs already discussed (Table II). On the other hand, the Compounds 4 and 5 produce satisfactory cholecystograms after intravenous injection. Compounds S and 6 produce clear visualization of the biliary ducts and gall bladders of dogs after both oral and intravenous administration. These results were corroborated by the fact that there were present in the livers of rats, at any period after their administration, larger amounts of Compound 6 than of the other compounds, including Compound S. This latter compound has been shown by Jones and coworkers(3) and in our own experiments to be an effective cholecystographic medium in dogs after either oral or intravenous administration.

Summary. 1. After intravenous injection into dogs, the following compounds were found to be good cholecystographic dyes: N-(3-amino-2,4,6-triiodobenzoyl) ε-amino-n-caproic acid (Compound 6); 2-(4-hydroxy-3,5-diiodobenzyl) benzoic acid (Compound S); 3-caproylamino-2,4,6-triiodobenzoic acid (Compound 5) and 3-butyrylamino-2,4,6-triiodobenzoic acid (Compound 4). After oral administration, only Compounds S and 6 produced satisfactory visualization of the gall bladder and biliary tract 16 hr after feeding. 2. Analyses of the tissues of rats after intravenous administration of the compounds (1-6 inclusive and S) gave the following results: In the liver, Compound 6 was present in greater concentration than any of the others tested; in the kidney, Compounds 3-formylamino-2,4,6-triiodobenzoic acid, 3-acetylamino-2,4,6-triiodobenzoic acid, 3-propionylamino-2,4,6-triiodobenzoic acid, and 3-butyrylamino-2,4,6-triiodobenzoic acid were present in high concentration during the initial intervals, but fell to a relatively low level at the end of 3 hr. It was found that the rates of excretion of these compounds by the kidney decreased with the increase in the length of the acyl group.

This study was made possible by the cooperation of the Mallinckrodt Chemical Works of Saint Louis. We are much indebted to Dr. Melvin A. Thorpe who made available compounds 1-6 and to Dr. W. H. Strain who generously placed Compound S at our disposal. Our thanks are also due to Dr. Thorpe for helpful advice during the course of the work.

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Effect of Certain B Vitamin Deficiencies on Gastric Secretion in the Rat.

(19060)

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Evidence has been accumulating for a number of years that nutritional deficiencies may impair the process of gastric secretion (1,2). Pellagra has been associated with a high incidence of achlorhydria. Goldsmith and associates(3) have observed recently the depression (absence in one) of hydrochloric acid secretion in two patients while on a pellagragenic diet with return of function when the diet was supplemented with nicotinic acid. In animal work, decreased acid secretion in vitamin deficient dogs has been observed; marked increases followed supplementation with a B complex preparation(4) or with yeast(5). Dyer and Roe(6) found that thiamine deficiency decreased acid secretion only when their rats were moribund, whereas Shay and associates(7) reported that this deficiency increased secretion.

The following experiments were conducted to obtain further information regarding the influence of certain specific B vitamin deficiencies on the secretory process.

Methods. Male rats of the Sprague-Dawley strain were received from the National Institutes of Health breeding colony at weaning (weight, 40-55 g; age, 21-27 days) and littermates were distributed equally to form groups of similar mean weight. The animals were housed individually in wire mesh bottom metal cages and had continuous access to tap water. Diet was supplied *ad libitum* to all except the pair-fed animals which were given their allotment of food each day before noon. Lack of water or food, or feeding the pair-fed animals late in the day, caused erratic results. With only a few exceptions, both *ad libitum* and pair-fed animals were used as controls to the deficient animals in each individual experiment. Satisfactory deficiencies developed in all experimental groups during our standard 3-week feeding period. Animals were considered deficient when they exhibited poor or no growth which could be definitely stimulated by a supplement of the particular vitamin which was absent from the diet. Evidence of early polyneuritis was present in the thiamine deficient animals at the end of the 3-week feeding period. Since nicotinic acid is synthesized in adequate amounts by the rat unless the dietary tryptophan level is relatively low, the low protein basal diet I (Table I)(8), less nicotinic acid, was used to produce a nicotinic acid deficiency. The corn-containing basal diet II

TABLE I. Basal Diets.

	I (%)	II (%)	III (%)
Casein "vitamin-free"	9	9	20
Cystine	.15	.15	—
Gelatin	3	—	—
Corn meal (unenriched)	—	40	—
Sucrose	80	43	72
Wesson oil	3	3	3
Salts O & M(9)	4	4	4
Vitamin mixture*	1	1	1

* Containing the following in mg/g: thiamine .4, riboflavin .6, pyridoxine .5, Ca pantothenate 4, choline chloride 200, inositol 20, nicotinic acid 4, 2-methylnaphthoquinone .2, biotin .02, and folic acid .2, in corn starch (Argo).

Vit A, D, and E as supplements: A and D 2000 and 400 U.S.P. units twice weekly. E 3 mg tocopherol once weekly.

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6. Dyer, Helen M., and Roe, Joseph H., *Am. J. Digest. Dis.*, 1941, v8, 329.

7. Shay, Harry, Komarov, S. A., Gruenstein, Margot, and Fels, S. S., *Gastroenterology*, 1946, v6, 199.

8. Hundley, James M., *J. Biol. Chem.*, 1949, v181, 1.

9. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, v32, 369.

(Table I), less nicotinic acid, was fed as another means of producing nicotinic acid deficiency. Basal diet I, less either thiamine or riboflavin, was used to produce the corresponding deficiencies with this type of diet. The complete vitamin mixture was included in the control diets. Deficiencies of thiamine, riboflavin, pyridoxine, and pantothenic acid were produced by feeding the higher protein basal diet III (Table I) from which the corresponding individual vitamins had been omitted. Control animals received the same basal diet containing the complete vitamin mixture.

As a further test of the effect of individual vitamin deficiencies, acute reversal experiments were done. Deficiencies of thiamine, riboflavin, pyridoxine, and pantothenic were produced by feeding basal diet III less the corresponding individual vitamin. Sixteen hours before pylorus ligation, food was withdrawn and half of each group was given by stomach tube an aqueous solution or suspension of the vitamin which had been omitted from the diet, while the other half was given a similar quantity of water. The accumulated gastric juice following pylorus ligation was used to study the effect of the various deficiencies. Our procedure was slightly modified from the method of Shay *et al.*(10). Food was removed from the cage at 5 p.m. so that the stomach would be empty the following morning at about 9 a.m. when the pylorus was ligated. Intraperitoneal sodium pentobarbital anesthesia (30 mg/kg), supplemented with ether when necessary, was used. Intra-abdominal trauma was minimized by gentle handling. The pylorus was manipulated with a small glass hook and ligated using care to avoid blood vessels. The abdomen was closed in two layers with the skin edges closely approximated so that covering the wound to prevent the animal from ingesting blood and serum was not necessary. The animal was returned to its cage, without water and still without food. At the end of our standard 6-hour secretory period,* timed from the operation, the animal was sacrificed with chloroform, care being taken to clamp off the esophagus as soon as possible to avoid loss of juice. The stomach was removed, the gastric juice expressed into a graduated centrifuge tube, and the stomach wall inspected. An animal was discarded for any one of the following reasons: failure to awaken from anesthesia within 1 hour, hemorrhage, perforation of an ulcer either into the peritoneal or the pleural cavity, excessive solids (over 0.5 cc) in the stomach contents, or grossly bloody juice.

After the volume had been measured the juice was centrifuged and aliquots of the clear supernatant were used for the determination of the concentration of free and total acid (dimethylaminoazobenzene and phenolphthalein, respectively) and pepsin (milk clotting method of Saemundsson)(12). Previously, two methods of comparing volume or rate of secretion have been used. In some types of work it has been possible to use animals of approximately the same size and merely compare the total volume secreted in a standard period of time(13). In other work, volume of secretion has been related to body size by the expression cc/100 g body wt/hr(10). This method is satisfactory, particularly if the size range of the animals is not excessive, and accordingly we have included in the tables figures for cc/100 g body weight (per 6 hrs). In addition, we have expressed volume of secretion as cc/100 sq cm body surface† (per 6 hrs). Using the latter method, we have obtained more constant figures for animals over a very wide weight range(15).

Results. Since the day to day variation for

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10. Shay, Harry, Komarov, S. A., Fels, Samuel S., Meranze, David, Gruenstein, Margot, and Siplet, Herman, *Gastroenterology*, 1945, v5, 43.

* After about 6 hours, volume of juice increases at a much slower rate and acute forestomach ulcers begin to appear(11).

11. Pauls, Frances, Wick, Arne N. and MacKay, Eaton M., *Gastroenterology*, 1947, v8, 774.

12. Saemundsson, Johann, *Acta med. Scand. Supp.*, 208, 1948.

13. Madden, Robert J., Ramsburg, Helen H., and Hundley, James M., *Gastroenterology*, 1951, v18, 119.

† Calculated according to the formula of Diack (14) as surface area $= kW^{2/3}$ using $k = 7.5$.

14. Diack, Samuel L., *J. Nutr.*, 1930, v3, 289.

15. Hawk, Edgar A., and Hundley, James M., *J. Nutr.*, 1951 in press.

TABLE II. Volume, Acid Concentration, Pepsin Concentration, and Pepsin Output for Control and Deficient Animals.

Deficiency	Basal diets	No. rats	Wt	Volume cc/100 g	Volume cc/100 cm²	Acid concentration* Free	Acid concentration* Total	Pepsin† mg/cc	Pepsin† mg/100 cm²
Control *ad lib.*	I	17	77	6.5 ± .2‡	3.7 ± .1‡	106 ± 2‡	123 ± 2‡	1 ± .03‡	3.6 ± .1‡
,, pr. fed	I	24	58	7.3 ± .1	3.6 ± .1	97 ± 2	117 ± 2	1 ± .04	3.5 ± .1
Nicotinic acid	I	24	50	7.8 ± .2	3.8 ± .1	106 ± 1	124 ± 2	.9 ± .03	3.5 ± .1
Thiamine	I	17	45	4.8 ± .2	2.3 ± .1	90 ± 4	113 ± 4	1.1 ± .05	2.6 ± .1
Riboflavin	I	18	55	5.9 ± .3	3 ± .1	103 ± 2	127 ± 2	1 ± .02	3.1 ± .2
Control	II	9	103	5.9	3.6	100	116	.8	3.1
Nicotinic acid	II	9	72	6.3	3.5	103	123	.9	3.1
Control *ad lib.*	III	53	108	6.3 ± .1	4 ± .1	100 ± 2	119 ± 1	.9 ± .02	3.4 ± .1
,, pr. fed	III	56	71	6.7 ± .1	3.7 ± .1	101 ± 1	125 ± 1	.9 ± .02	3.2 ± .1
Thiamine	III	27	60	5.4 ± .2	2.8 ± .1	86 ± 4	109 ± 3	1.1 ± .03	3.2 ± .1
Riboflavin	III	27	58	6.7 ± .2	3.4 ± .1	105 ± 3	124 ± 2	.9 ± .02	3.1 ± .1
Pyridoxine	III	21	66	5.5 ± .2	2.9 ± .1	81 ± 2	112 ± 2	1.5 ± .1	4.2 ± .2
Pantothenic acid	III	22	70	6.1 ± .2	3.3 ± .1	107 ± 1	129 ± 1	.9 ± .02	3 ± .1

* Milliequivalents/liter. † As activity equivalent to crystalline pepsin (Armour & Co.). ‡ Probable error of the mean.

the various groups was not significant, the data for all animals in similar experimental groups have been combined in Table II.

Nicotinic acid deficiency did not reduce the volume of secretion. The figures for cc/100 sq cm are almost identical for deficient and control groups. If expressed as cc/100 g, the deficient animals secreted more than did the controls.

Both thiamine and pyridoxine deficiencies sharply reduced the volume of secretion below that of the control animals. The differences between the performances of deficient and control animals were apparent whether expressed as volume per unit of body weight or per unit of surface area and were statistically highly significant.‡

Riboflavin and pantothenic acid deficiencies were accompanied by moderate depressions in volume of secretion when this was expressed as cc/100 sq cm. The differences were of borderline statistical significance. When volume was expressed as cc/100 g, the differences were less definite or even not present as in the case of the riboflavin deficient animals on basal diet III.

In the acute reversal experiments (Table III) there was an insignificant rise in the volume of secretion following supplementation with the missing vitamin in the thiamine and pantothenic acid deficient animals and no change in the riboflavin deficient group. However, in the pyridoxine deficient group, there was a marked rise in volume of secretion in the supplemented animals.

Pepsin concentrations in the nicotinic acid, riboflavin, and pantothenic acid deficient groups were approximately equal to the control values (Table II). In both thiamine deficient groups, pepsin concentration was very slightly elevated but total output was moderately depressed in the basal diet I animals and was very near the same as that for the controls in the basal diet III animals. However, in the case of the pyridoxine deficient animals both pepsin concentration and total output were definitely above the con-

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‡ Statistical significance of differences was determined according to the test procedure described in *Chemistry of Food and Nutrition,* Henry C. Sherman, 7th ed., The Macmillan Co., New York, 1946.

TABLE III. Volume, Acid Concentration, Pepsin Concentration, and Pepsin Output in Deficient Animals After a Single Administration of the Missing Vitamin.

Deficiency	Supplement	No. of rats	Vol, cc/100 cm²	Free acid[*]	Pepsin,[†] mg/cc	Pepsin. mg/100 cm²
Thiamine	Water	16	2.9 ± .1[‡]	91 ± 5[‡]	.9 ± .02[‡]	2.7 ± .1[‡]
	100 μg thiamine	16	3.2 ± .1	88 ± 4	.9 ± .03	3 ± .1
Riboflavin	Water	15	3.8 ± .1	103 ± 2	.9 ± .03[§]	3.1 ± .1
	100 μg riboflavin	16	3.9 ± .1	112 ± 2	.9 ± .03[§]	3.3 ± .1
Pyridoxine	Water	16	3 ± .1	90 ± 2	1.2 ± .03	3.4 ± .1
	100 μg pyridoxine	15	4.3 ± .1	116 ± 2	.9 ± .03	4.2 ± .2
Panto. acid	Water	22	3.5 ± .1	110 ± 2	.8 ± .02[‖]	2.7 ± .1
	1 mg Ca panto.	23	3.9 ± .1	109 ± 1	.8 ± .02[‖]	3.1 ± .1

[*] Milliequivalents/liter.
[†] As activity equivalent to crystalline pepsin (Armour & Co.).
[‡] Probable error of the mean.
[§] 8 determinations. [‖] 15 determinations.

TABLE IV. Effect of Addition of Vitamin B_{12} and/or Aureomycin to Control Diet (Basal III).

Addition	No. of rats	wt	Vol, cc/100 cm²	Free acid concentration[*]	Pepsin,[†] mg/cc
Control	10	114	3.9	106	.8
Vit. B_{12} (5 μg %)	9	125	3.9	96	.8
Aureomycin (20 mg %)	9	133	3.9	105	.8
Vit. B_{12} (5 μg %) and aureomycin (20 mg %)	10	136	3.9	101	.7

[*] Milliequivalents/liter.
[†] As activity equivalent to crystalline pepsin (Armour & Co.).

trol levels. In the acute reversal experiments, pepsin concentration fell with pyridoxine supplementation but the total amount secreted actually rose, tending to follow the increase in volume of secretion. The concentration of free and total acid varied but little in any of the groups, although the values for the thiamine and pyridoxine deficient animals were slightly below control levels (Table II).

Except in the thiamine and riboflavin deficient animals, the stomachs showed no gross changes other than an occasional acute forestomach ulcer which did not appear to be related to the nutritional status of the animal. In many of the thiamine deficient animals, we noted the presence in the glandular portion of the stomach of the acute hemorrhagic ulcers previously described by Shay *et al.*(7). Grossly similar but fewer and less severe lesions were seen in the stomachs of the riboflavin deficient animals.

Since none of the diets used in the experiments reported above contained added vit B_{12}; and in view of the current interest in antibiotics, additional groups of animals were fed basal diet III modified by the addition of either crystalline aureomycin, 20 mg %[§]; vit. B_{12}, 5 μg %; or both. These animals were slightly larger than the controls at the end of the standard feeding period, as shown in Table IV, but changes in volume of secretion, acid and pepsin concentration, and total output of pepsin were very small and definitely not significant.

Discussion. The nicotinamide-containing DPN or coenzyme I has been strongly suspected as a participant in the acid secretory process(16). Or inability to obtain evidence of impaired acid secretion in nicotinic acid deficiency does not rule out an important role for DPN in the process of secretion since the coenzyme level in the particular cells concerned may have been maintained above the level necessary for normal function.

Our results with thiamine deficient animals differ from those of Shay *et al.*(7) who reported increased secretory rates in this deficiency. However, this difference may be explained as resulting from the very different technics employed. Whereas our animals were started from weaning and maintained for 3 weeks on the deficient diet, those of Shay *et al.* were larger when started on a deficient diet and were maintained until considerable weight losses had occurred. Furthermore, we employed only pylorus ligation with collection of total gastric contents 6 hours later, whereas the other workers used pylorus ligation plus a fistula for juice collection at intervals over a longer period of time, with fluids being given as required. A third difference was that our animals were out of anesthesia shortly after the operation while those of Shay *et al.* were kept anesthetized during much of the secretory period. Our results also differ from those of Dyer and Roe(6) who found that thiamine deficiency decreased the secretory rate only when their animals were moribund. While our animals were definitely deficient, they were not moribund. In view of the marked circulatory disturbances frequently observed in thiamine deficiency, it seems possible that the observed depression in secretion may be at least partially explained on the basis of circulatory depression and thus may not be entirely a direct biochemical effect on the secretory mechanism.

[§] Kindly supplied by Drs. T. H. Jukes and E. L. R. Stokstad of Lederle Laboratories.

16. Patterson, W. B., and Stetten, DeWitt Jr., *Science*, 1949, v109, 256.

The sharp depression in volume of secretion in the presence of pyridoxine deficiency is evidence that this substance plays a previously unsuspected role in the secretory process. Even better evidence for the involvement of this vitamin is the observation that secretion can be returned to a high level by means of a small supplement of pyridoxine alone given about 16 hours before the start of the secretory test. The rapidity of this effect and the fact that the test was conducted with no possibility of further access to food increases the probability of a specific role for pyridoxine in gastric secretion. The reason for the increased pepsin concentration and output in the pyridoxine deficient animals is not apparent at this time. The small depressions in volume produced by the riboflavin and pantothenic acid deficiencies and the slight changes observed in the acute reversal experiments do not constitute impressive evidence implicating these vitamins in the secretory process.

The correlation of both free and total acid concentrations of the juice with volume secreted is compatible with the accepted theory that acid is secreted by the parietal cells as a constant mixture. The small reductions in acid concentration observed in a few of our experiments could be explained on the basis of a relatively greater reduction in secretion of the acid component of the juice as compared with secretion of non-acid components and with salivary contamination.

Although vit. B_{12} was not added to the diets routinely, it is obvious from the results obtained when this substance was included that our experiments were not complicated by an unrecognized deficiency of vit. B_{12}.

It may be noted that, although the vitamin deficiencies produced were quite severe, achlorhydria such as has been reported in human deficiency diseases was not seen in any of the animals. This difference may be explained by the fact that our experiments were conducted in young animals over a short period of time and multiple vitamin deficiencies were not present.

Summary. 1. The influence of various individual B vitamin deficiencies on gastric secretion has been investigated in the pylorus ligated rat. Pyridoxine deficiency sharply reduced acid secretion. This effect was completely reversed by pyridoxine given only 16 hours before the start of the secretory test. Thiamine deficiency markedly depressed acid secretion. Nicotinic acid deficiency did not depress secretion, while riboflavin and pantothenic acid deficiencies produced only small depressions. 2. Neither aureomycin nor vit. B_{12} had any effect on gastric secretion when added to the complete basal diet, although each increased weight gain slightly. 3. Pepsin secretion was not markedly depressed in any of the deficiencies studied, with the possible exception of thiamine. Pyridoxine deficiency increased both concentration and total output of pepsin.

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Biotin Deficiency and Cryptorchidism in Rats.* (19061)

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A form of cryptorchidism attributed to biotin deficiency was recently reported in rats (1). The reproductive anomaly was produced in 8 rats in 2 experiments involving a diet rich (66%) in spray-dried egg white, containing avidin, a protein which combines with biotin within the intestine in a non-absorbable complex(2). The cause of the cryptorchidism was suggested as due to the contracture of the cremaster muscle which completely surrounds the testicle in the rat. Biotin deficiency symptoms have been described in detail in the rat(3) and fowl(4), and the biochemical action of this vitamin in carboxylase and deaminase systems has been reported in bacteria and mammalian tissues(5,6). Although other vitamin deficiencies, for example, A and E, have been involved in male reproductive disorders, the claim that biotin deficiency may specifically cause testicular ascent is of unusual interest. In the absence of details pertaining to biotin therapy, contractility of the cremaster muscle, or growth and weight changes in the experimental animals, we have attempted to repeat and elaborate on this work.

Five experiments, involving 98 male weanling albino rats, were conducted to test the specific effect of biotin deficiency in causing cryptorchidism, as well as possible factors mediating or counteracting the reproductive disorder. Rats of the University of Massachusetts and the Carworth Farms strains were used in all tests. The animals were isolated in wire screen cages with screen bottoms and fed weighed quantities of food: 10 to 15 g/rat/day or as much as each animal would eat, with water supplied *ad libitum*. Litter mate controls were used where possible. Weanling weights, however, varied considerably, between 29 and 48 g. The experiments and conclusions are summarized in Table I.

Exp. I. Rats, placed on a commercially-prepared biotin-deficient diet‡ 8 days after weaning, continued to gain weight and appeared normal for a period of 7 weeks. Following this, growth was subnormal, and finally the characteristic, but not unique, symptoms of biotin deficiency developed, including scaly eyelids, desquamation of the skin, falling hair, arched back, and, within 3 months, the anticipated cryptorchidism. These symptoms did not appear in controls fed Purina Laboratory Chow and were remedied in those animals returned to chow after 3 months on the experimental ration. A characteristic deficiency symptom—a marked protrusion of the penis—in these and the semi-starved rats (Exp. V) appeared shortly before death.

Exp. II. Animals placed on the basal biotin-free diet immediately after weaning became cryptorchid within 20 to 25 days, but the marked severity of this ration was demonstrated by its lethal effect in allowing a survival period of 36 days (average) after weaning. The symptoms of biotin deficiency which developed were similar to, and masked by, symptoms of severe inanition and starvation. The addition of excessive egg white to the basal diet, at various times after the initiation

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‡ S-7 formula of Welsh and Wright(7) prepared by General Biochemicals, vitamin test casein 18, hydrogenated vegetable oil 10, corn oil 2, sucrose 59.9, salt mixture 4, non-nutritive fibre 4, vit. A, D and E concentrate .08, choline chloride .10 (g); thiamine hydrochloride .2, riboflavin .4, pyridoxine hydrochloride .2, nicotinic acid 4, calcium pantothenate 4.4, p-aminobenzoic acid 4, inositol 8 (mg).

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TABLE I. Summary of Biotin-Deficiency Diets, Responses, and Effects of Supplements Administered to Weanling Albino Rats.

Group		Diet	Supplement	Response
I A	(6)[*]	Biotin-dr[‡] (S-7) beginning 8 d post weaning		C[¶] within 3 mo
B	(3)	Laboratory Chow (control)		Descended testes
II A	(4)	Biotin-dr (S-7)		C (<25 d) dead in 36 d
B	(9)	dr + 20% egg white, beginning 27th day		C (20-25 d) dead in 38 d
C	(7)	dr + 20% egg white, beginning 6th day		C (20-25 d) dead in 36 d
D	(8)	dr + 20% egg white, beginning immediately		C (20-25 d) dead in 33 d
E	(4)	dr	Biotin[†] fed 100 μg/d	C
F	(4)	dr	” inj. 150 μg, 3x/wk	C
III A	(5)	Biotin-dd[‖] (9-A) + 20% egg white		Descended testes
B	(5)	dd + 40% egg white		” ”
C	(5)	dd + 20% egg white	Biotin inj. 150 μg, 3x/wk	Descended testes. Increase in body wt
IV A	(6)	dr (S-7)		4 C. 2 testes small (40 d)
B	(4)	dr	Testosterone[§] inj. 500 μg, 2x/wk	C
C	(10)	dr	Chorionic gonadotrophin[§] 50 units, 2x/wk	Descended testes
D	(4)	Laboratory Chow (control)		” ”
E	(4)	” ” ”	”	” ”
V A	(5)	” ” (½ ration)		Descended testes after delay
B	(5)	” ” ”	”	Normal testes

[*] No. of animals.

[†] The biotin (free acid) was obtained from General Biochemicals, Inc.

[‡] dr = deficient ration.

[§] Testosterone propionate (Oreton) and chorionic gonadotrophin (Pranturon) contributed by the Schering Corp.

dd = deficient diet.

[¶] C = cryptorchid.

of the experiment, caused little change except perhaps to shorten the life span (II, B,C,D). Two groups of animals administered biotin in the food (II, E) or by intraperitoneal injection (II, F) throughout the experiment showed no improvement whatever in the cryptorchid condition. Biotin injected rats, however, lived longer than animals not receiving biotin (46 compared with 36 days on the average). Because of the immediacy of response to this severe dietary regimen and the lack of effective biotin therapy, the tentative conclusion was drawn that inanition rather than biotin deficiency *per se* was the cause of the cryptorchidism.

Exp. III. The severity of the dietary response elicited in the previous experiments suggested the use of a milder biotin-deficient ration. As several of the components of Manning's diet(1) were unavailable, we used a diet previously shown to be biotin-free in extensive experiments with mice(8). On this ration,[§] begun at weaning, our rats did not develop cryptorchid symptoms (III, A). To assure further the requirements of a biotin-free diet, the egg white content was increased from 20% to 40%, without producing crypt-

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8. Wilson, J. W., Leduc, E. H., and Winston, D. H., *J. Nutrition*, 1949, v38, 73.

orchidism (III, B). However, vitamin deficiency was indicated by the increase in body weight toward normal in those rats injected with biotin (III, C).

Exp. IV. The usual association of cryptorchidism with hypophyseal malfunction suggested that this gland might be involved, particularly in relation to the secretion of gonadotrophin. A decrease in pituitary stimulation, as opposed to a reduction in responsiveness of the tissues, might be expected to result from such a dietary deficiency. Ten animals were placed on the severe S-7 biotin-deficient diet at weaning and given chorionic gonadotrophin intraperitoneally (50 units twice weekly) beginning at weaning. In all 10 rats the testes descended within 10 to 12 days (IV, C). The positive response to gonadotrophin was temporary, however, and within a week the testes began to reascend. While the gonadotrophin thus appeared to counteract or mask the cryptorchidism, therapeutic attempts with testosterone propionate (500 μg twice weekly) proved ineffective (IV, B). Laboratory Chow controls showed the same testicular development (IV, D) as did rats fed chow while receiving chorionic gonadotrophin (IV, E).

Exp. V. The effect of slow starvation with a reduced quantity of complete diet was tested by feeding Laboratory Chow in amounts up to one-half the normal intake While not unequivocal, these results indicate that testicular development may be retarded in semi-starved rats, smaller gonads descending 1-2 weeks after those of controls and then with signs of reduced spermatogenesis (V, A,B). Supplements of gonadotrophin administered along with this semi-starvation diet partially remedied the incipient cryptorchidism.

Some rats from each experimental group, while under nembutal anesthesia, were investigated for contractility of the cremaster musculature. Isotonic contractions recorded kymographically from the tip, the body, or from an excised strip of cremaster muscle showed no characteristic differences in the control and experimental animals other than those due to size of the animals. Weak responses in rats which had undergone severe dietary restriction and inanition indicated that lesser, rather than greater, contractility of the cremaster muscle occurred.

These results are interpreted to indicate that the cryptorchidism is brought on by a type of pseudo-hypophysectomy resulting from general inanition and is not specifically induced by biotin deficiency(9). Biotin lack may intensify the condition, but is not the sole cause of the cryptorchidism, nor is biotin therapy effective in remedying the situation.

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§ 9-A ration of Wilson *et al.*(8): vitamin test casein 10, egg white 20, corn starch 28.5, Crisco 20, corn oil (Mazola) 4, cod liver oil (Squibb) 2, powdered brewer's yeast 8, salt mixture U.S.P. XII_2 7, Sulfaguanidine .5 (g) Egg white supplied by Henningsen Lamesa, Inc.; sulfaguanidine contributed by Lederle Laboratories.

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An Attempt to Transmit Rheumatoid Arthritis to Humans. (19062)

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The etiology of rheumatoid arthritis remains an enigma. The suppressive effects of cortisone on the manifestations of the disease throw no light on its primary cause. Because of this, and because of the many features of the disease which suggest an infectious etiology, such as fever, leucocytosis, increased sedimentation rate, weight loss and the signs of joint inflammation, a return to the search for infection seems indicated. This search however, must be at the level of the ultramicroscopic organisms since many years of intensive research have excluded a bacterial cause. The recent isolation of pleuropneumonia-like organisms in Reiter's Syndrome, which closely resembles rheumatoid arthritis

(1,2) and the beneficial effects of antibiotics in certain cases of rheumatoid arthritis(3), strengthen the hypothesis of an infectious cause. Gordon suggests that there may be a group of "rheumatic" viruses having specific affinity for connective tissue structures. He observed elementary bodies in rheumatic tissues, and drew attention to the highly specific affinity of viruses for particular tissues such as the dermatotrophic smallpox virus and the neurotrophic poliomyelitis virus(4).

The following study was undertaken to explore the possibility of virus etiology. Since viruses (such as the virus of infectious hepatitis) may be species-specific, an attempt at direct human passage was the method chosen.

Procedure. Five subjects exhibiting the classical features of active rheumatoid arthritis and having effusions into joints or bursae were selected as donors. Under sterile conditions with local procaine anesthesia approximately 10 cc of fluid was aspirated from the involved joint or bursa of each donor. A sample of this fluid was submitted to routine bacteriologic study and was in all instances sterile. The fluid was collected in sterile rubber stoppered vials and stored at −20°C until an appropriate recipient was available. The group of recipients consisted of 10 males and 7 females, aged from 42 to 87 years, the average age being 61.2 years. They were chosen because their prognosis for life beyond several weeks was considered hopeless. Their illnesses were as follows: Six cases of various kinds of metastatic carcinoma, one case of myelogenous leukemia, 4 cases of malignant hypertension, one case of severe recurrent embolism of cardiac origin, 2 cases of severe arteriosclerotic gangrene, one case of renal failure with uremia and 2 cases of terminal hypertensive heart disease. Under sterile conditions, and local anesthesia with procaine, 1.0 cc of the joint fluid obtained from the donor subject was injected directly into one of the knee joints of the recipient subject. The injected joint was examined at one to 3 day intervals for evidence of local disease, and the patient was observed for evidence of systemic infection or reaction. The post-injection periods of observation ranged from 2 days to 92 days, the average period of observation being 28.7 days. Two of the patients were observed for 92 days and 85 days respectively, and 8 patients were observed for periods of longer than one month's duration.

Results and discussion. In 3 instances transient mild local warmth was noted in the injected joint of the recipient subjects. This occurred within 24 to 48 hours after injection and was of only 24 to 36 hours duration. There was no associated swelling or discomfort and it was felt that this simply represented a local irritant or foreign body type of reaction. In no case was there any real articular or systemic manifestation suggesting any form of arthritis.

Four cases of the series were subjected to post-mortem examination. In none of these was there any histologic evidence of articular or periarticular inflammatory changes which could indicate transmitted infection.

The negative findings by the method as above described do not by any means exclude the possibility of a virus etiology of rheumatoid arthritis, for the following reasons: 1. An element of natural or acquired immunity cannot be excluded since most of the recipient subjects were in the older age group. 2. An associated stress factor may be necessary for the precipitation of active clinical disease, the virus or other causative agent remaining dormant unless this occurs. 3. Because of the long latent periods characteristic of certain viruses, notably that of serum hepatitis, the periods of observation in this study may be too short for the disease to develop.

Conclusions. In no instance was there clinical or pathological evidence for transmission of active rheumatoid arthritis from one human to another by the direct passage method of study as described.

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Fibrinoid and Hydroxyproline of the Rheumatoid Subcutaneous Nodule.* (19063)

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In a number of diseases which have been designated as "collagen diseases", degeneration of collagen with replacement by a fibrinoid material is described. Fibrinoid has been variously considered to be fibrin(1,2), degenerated collagen(3,4) and a complex between degenerated collagen and mucopolysaccharide(5).

The amino acid, hydroxyproline, constitutes approximately 14% of collagen as derived from a variety of sources(6), and except for the small amounts present in elastin, is found in no other protein of the body. In view of the fact that fibrinoid has been described as a degenerated form of collagen, it was of interest to determine whether the total hydroxyproline content of rheumatoid subcutaneous nodules, which contain relatively large amounts of fibrinoid material, could be accounted for entirely in the collagen and elastin fractions. Three subcutaneous nodules, containing considerable amounts of fibrinoid material as demonstrated by histological examination of tissue sections, were analyzed. It was found that the hydroxyproline content of the collagen fraction of each nodule corresponded closely to the recorded values of Neuman and Logan(6) for purified collagen from a number of sources, and that the total hydroxyproline content of the nodules was accounted for entirely by the collagen and elastin fractions.

Experimental. Nodules were excised under local procaine anesthesia. A portion of the nodule was reserved for histological examination.[‡] The remaining tissue, varying in weight between 1 and 5 grams, was minced finely and divided into several portions. Extractable lipid was determined by a single extraction with a solution of 3 volumes of alcohol and 1 volume of ether, followed by three extractions with ether. Dry weight was determined following fat extraction by drying at 105°C for twenty-four hours. Repeated determinations showed that constant weight was achieved by the drying procedure. Collagen and elastin were analyzed on 0.5 to 1 gram samples of tissue by the method of Lowry, Gilligan and Katersky(7), the collagen being determined on the basis of the nitrogen content of the gelatin supernatant and calculated on the basis of 17.5% nitrogen in collagen. Hydroxyproline was determined by the method of Neuman and Logan(6). The hydroxyproline content of collagen was determined in the gelatin solution obtained in the procedure of Lowry *et al.*(7). Total hydroxyproline content was determined by hydrolyzing approximately 0.1 to 0.3 gram of minced tissue in 1 ml of 6 N hydrochloric acid for four hours in a sealed tube at fifty pounds pressure, then neutralizing and diluting to appropriate volume. There was no interfer-

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TABLE I. Analysis of Rheumatoid Nodules and Synovia.

Tissue	% water	% lipid	Collagen as % wet wt	Hydroxyproline as % collagen	Elastin as % wet wt	Hydroxyproline as % wet wt of tissue	% Hydroxyproline* from collagen and elastin
Nodule B	80.6	2.44	10.1	14.7	0.52	1.41	1.49
Nodule S	85.8	1.86	7.08	13.9	0.43	1.11	0.992
Nodule K	72.6	2.46	13.5	12.8	1.70	1.72	1.76
Synovial membrane	72.8	2.93	17.4	13.9	1.29	2.63	2.44

* Calculated on the basis of 1.8% of hydroxyproline in elastin(6).

ence with color development, and the absorption spectrum obtained (between 500 and 600 mμ) corresponded quantitatively with that of a standard solution of hydroxyproline. When 3.0 mg of hydroxyproline were added to duplicate samples of 0.2 gram of minced tissue which were subsequently hydrolyzed in 6N hydrochloric acid as described above, 3.0 and 3.1 mg, respectively, were recovered.

Results. Water, collagen and lipid comprised 90 to 95% of the net weight of the 3 nodules as well as of the synovial membrane (Table I). Whereas the nodules and the synovial tissue were relatively rich in collagen, the elastin content of these tissues was low. This fact coupled with the low hydroxyproline content of elastin indicates that elastin contributes negligibly to the total hydroxyproline content of the tissue. The values of 13.9, 14.7 and 12.8% respectively found for the hydroxyproline content of the collagen of the 3 rheumatoid nodules, and that of 13.9% in the synovial membrane are in fair agreement with recorded values observed in samples of normal collagen derived from a variety of sources(6,8), and suggests that there is no significant variation in the composition of the collagen of the subcutaneous nodule.

Camera lucida tracings of representative sections of nodules S and B indicated that the areas occupied by fibrinoid material were approximately 25 to 26% as large as the areas occupied by collagen. This was demonstrated by a group of staining procedures including the van Gieson and Trichrome stains. If fibrinoid were a protein of collagenous origin, one might reasonably expect that it would contribute significantly to the total hydroxyproline content of the nodule tissue, unless, of course, the change of collagen to fibrinoid involved the loss of hydroxyproline specifically from the protein chain. The latter, though it is unlikely, cannot be ruled out.

Fibrinoid is extracted from subcutaneous nodule tissue(9) during the preliminary treatment with 0.1 N sodium hydroxide in the procedure of Lowry *et al.* for the determination of collagen and elastin. If fibrinoid contained hydroxyproline in the amounts present in collagen, one would expect significant differences between the total hydroxyproline content of nodule tissue obtained by analysis of the wet tissue (expressed as % wet weight in Table I) and the value calculated from the collagen and elastin contents (Table I). With experimental errors due to small samples taken into account, there is no large discrepancy in the % hydroxyproline arrived at by the two procedures. This would preclude the loss of significant amounts of hydroxyproline on alkaline extraction and indicates that fibrinoid, since it is removed by such treatment, does not contain hydroxyproline, at least in amounts approaching that in collagen. The latter observation would suggest that the fibrinoid material of the rheumatoid nodule is not of collagenous origin. Further quantitative studies are in progress(9).

Summary. Three subcutaneous nodules and one specimen of synovial membrane from patients with rheumatoid arthritis have been subjected to analysis for water, lipid, collagen, elastin and hydroxyproline. The hydroxyproline content of the collagen in these tissues was similar to that found in collagen from

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other sources. The total hydroxyproline content of the nodules and the synovial tissue was accounted for completely by the collagen and elastin fractions. The suggestion is made on this basis that the fibrinoid material of the rheumatoid nodule is not of collagenous origin.

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The *In vitro* Interchange of Cholesterol Between Plasma and Red Cells.* (19064)

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In previous studies on the incorporation of C^{14}-acetate carbon into cholesterol in various tissues of intact animals(1), it was observed that newly synthesized cholesterol appeared in liver, plasma, and red blood cells with surprising rapidity and attained uniform distribution within a few hours. Further investigation in dogs led to the conclusion that a rapid interchange of cholesterol must be assumed to occur in the living animal to explain the rapidity of equilibration of isotopic cholesterol between liver, plasma, and red cells(2,3). It therefore seemed of interest to investigate cholesterol interchange *in vitro*. Hahn and Hevesy explored the *in vitro* interaction between P^{32}-labeled phospholipids of plasma and red cells and found a very limited interchange—5% in 4.5 hours in rabbit blood(4). Although only total phospholipid P was measured, these authors concluded that some phosphatides exchanged with great speed and others not at all.

It is now generally accepted that the phospholipids and cholesterol of plasma are present in lipoprotein form and this would appear to be true also for red cells. However, the unesterified cholesterol moiety of both plasma and red cell lipoproteins is chemically identical and is therefore particularly suitable for interchange studies.

Procedure and results. Whole blood containing C^{14}-labeled cholesterol of high specific activity was obtained from a dog previously maintained on a cholesterol-free diet by the oral administration of 100 to 400 μc of C^{14} as carboxyl-labeled acetate, followed by the exsanguination of the animal about 24 hours later. The blood was treated with an anticoagulant (A.C.D. solution or oxalate) and cells and plasma separated. The interfacial layer was rejected and the cells were washed 3 times with isotonic salt solution. Unlabeled dog blood was drawn and treated in the same way except that the cells were not washed. In most experiments labeled cells and labeled plasma from one dog were each incubated with their unlabeled counterparts from a normal dog. In one experiment, normal human red cells were incubated with radioactive dog plasma. Measured volumes of red cells and of plasma were mixed and incubated at 37°C with gentle agitation and a slow stream of 95% oxygen plus 5% carbon dioxide was passed through the flasks. Aliquots were removed at intervals up to 4 hours and centrifuged. The interfacial layer was rejected and total lipid extracts were prepared from aliquots of plasma and red cells by 3 extractions with hot alcohol-ether (3:1), to a total of 10 volumes of solvent. Aliquots of the clear lipid extract were analyzed for free and total cholesterol by the Sperry - Schoenheimer

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* Supported in part by the Otho S. A. Sprague Memorial Institute and in part by contract AT(11-1) 56 with the Atomic Energy Commission.

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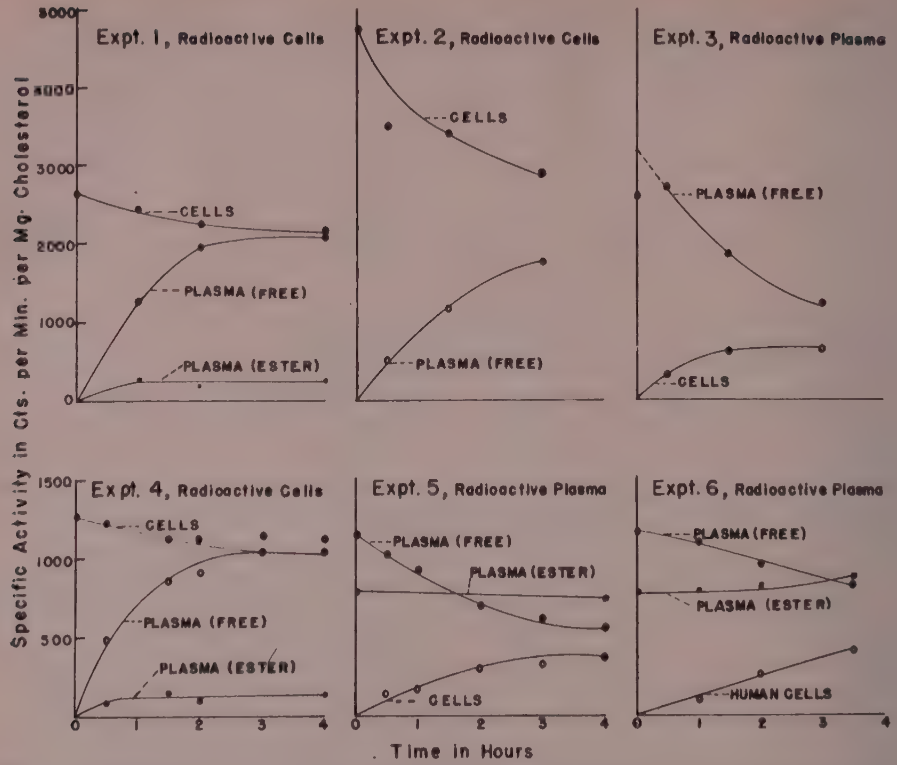

FIG. 1. Equipartition of C^{14}-cholesterol between cells and plasma. The specific activity which is proportional to the concentration of labeled cholesterol in the total cholesterol is plotted against time. In Exp. 6, human cells were incubated with dog plasma, and in all other experiments dog plasma and cells were used.

method as recently described(5). Larger digitonide samples were prepared for C^{14} assay by essentially the same procedures except that only a slight excess of digitonin was used. The assay technic is described elsewhere(6). All specific activities are expressed in terms of counts per minute for an infinitely thick layer of cholesterol (arbitrarily defined as counts per minute per mg cholesterol).

Fig. 1 shows the results of all 6 experiments, the cholesterol specific activities being plotted against time. It is evident that interchange of labeled cholesterol was very rapid in both directions. The total decrease in specific activity of the labeled component varied in each experiment depending on the relative masses of free cholesterol in the 2 phases.

Specific activities of esterified cholesterol in plasma were calculated from the specific activities and analyses for free and total cholesterol. Fig. 1 shows that esterified cholesterol of plasma neither acquired significant quantities of isotopic cholesterol (Exp. 1 and 4) nor did the labeled cholesterol ester fraction show any significant decrease in specific activity (Exp. 5 and 6). From this it may be concluded that esterified cholesterol was not capable of exchanging and also that the cholesterol esterases were not active under the conditions used. In the intact animal, however, cholesterol esters have been found to come to equilibrium with the free plasma cholesterol.

Analyses on all aliquots showed no significant change during incubation. Table I gives

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TABLE I. Cholesterol Analyses and Total Isotopic Cholesterol Content.

			Plasma component			Red cell component		Total C^{14} in cholesterol†	
				Cholesterol level					
Exp. No.	Labeled component	C^{14}-blood from dog No.	Vol, ml	Free, mg %	Ester, mg %	Vol, ml	Cholesterol level, mg %	Initial $\times 10^{-3}$	Final $\times 10^{-3}$
1	Cells	1	25	31	80	25	152	99.8	97.6
2	,,	2	22	27	79	14	155	99.4	84.9
3	Plasma	2	34	30	64	14	147	32.7	29.2
4	Cells	3	50	37	91	50	131	82.4	86.6
5	Plasma	3	50	62	148	34	152	35.7	36.5
6*	,,	3	48	62	148	20	92	33	35.6

* Radioactive dog plasma was incubated with human red cells.

† Total C^{14} in cholesterol was obtained by multiplying the cholesterol specific activity (c/m/mg) by total mg for each component and summating.

Bloods from dogs No. 1 and 2 were collected into A.C.D. solution so that the plasmas were diluted considerably. Potassium oxalate crystals (.2 g/100 ml) were used as anticoagulant in the blood from dog No. 3.

average values for each experiment.

Both labeled red cells and plasma contained C^{14}-labeled constituents other than cholesterol. Such labeled compounds will not interfere with the cholesterol studies unless they are converted into cholesterol during the incubation. Estimates of the total amount of isotopic cholesterol present in each incubation mixture did not show any significant change during the experiment as shown in Table I. It is of interest to note that cholesterol had a considerably higher specific activity than any other constituent examined.

As the curves in Fig. 1 appear similar to those for a first order process the data have been plotted in exponential form. In Fig. 2 $\log \frac{a}{a-x}$ is plotted against time, *a* being the final equilibrium specific activity for each experiment (obtained by extrapolation) and *x* being the change in specific activity from the initial value to the value at time *t*. In this way both ascending and descending specific activity curves can be plotted on the same basis. A close approach to linearity was shown by each of the individual experiments. Average values for all 5 dog experiments are shown in Fig. 2. The time required to reach 50% equilibration was 1.1 hours for red cells and 0.95 hour for plasma or an average of very close to 1 hour.

The single experiment with dog plasma and human red cells gave similar results (Fig. 1, Exp. 6), showing that the phenomenon is not species specific. The rate appeared somewhat slower but additional experiments will be necessary to determine if this difference is significant.

Discussion. During the last 20 years the concept that cholesterol and phospholipids are bound to specific proteins as lipoproteins has been developed and has recently been given strong support by the isolation and characterization of purified β_1 lipoprotein by Oncley, Gurd, and Melin(7). The nature of the link-

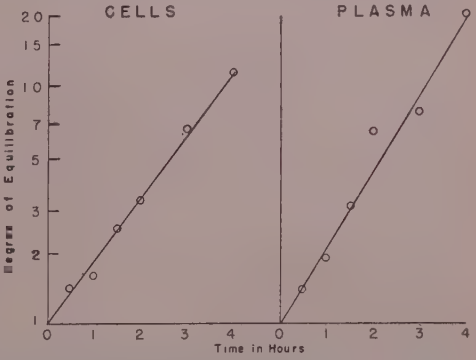

FIG. 2. The degree of equilibration of C^{14}-cholesterol between red cells and plasma expressed as $\frac{a}{a-x}$, plotted on a logarithmic scale against time. Each point represents the average of all data for that time interval for the movement of labeled cholesterol both into and out of dog red cells (on the left) and plasma (on the right). Data from the human red cell experiment are not included. Equilibration is half completed when $\frac{a}{a-x} = 2$, which is at approximately one hour in each case.

age between lipids and proteins is not understood but it has been generally considered to by a stoichiometric linkage with some stability rather than an adsorption complex. The experiments reported in the present paper show that unesterified cholesterol molecules initially present in plasma lipoproteins interchange with those in red cell lipoproteins during incubation. It appears highly unlikely that the entire lipoprotein molecules interchange since plasma lipoproteins contain both free and esterified cholesterol whereas red cells contain only the free form. Furthermore, red cell lipids occur in the water insoluble stroma in contrast to the highly water soluble plasma lipoproteins. All the free cholesterol molecules of plasma and of red cells are apparently capable of interchange. Although the radioactive cholesterol molecules measured in these experiments were recently synthesized, there is no reason to believe that a plasma lipoprotein molecule containing newly synthesized cholesterol will behave any differently from one containing "older" cholesterol. In the case of red cells, the fact that complete equipartition with free plasma cholesterol is attained both *in vivo* and *in vitro* is strong evidence for the assumption that all the free red cell cholesterol is exchangeable. The time required to reach 50% equipartition can be seen from Fig. 1 or 2 to be about one hour, corresponding to a turnover time of 1.44 hours. Thus, any given sample of plasma will have a minimum of 1.16% of the free cholesterol present leaving each minute and being replaced by an equal amount of red cell cholesterol. For example, in a liter of average blood the total cholesterol exchanged between plasma and red cells would be about 460 mg per hour.

The mechanism of this exchange is not simple diffusion since equalization in the free cholesterol concentrations of the 2 phases does not occur. The simplest assumption is that equilibria exist between cholesterol in each of the various lipoproteins concerned and cholesterol in unbound form. This last might be present in minute concentration, possibly in true aqueous solution, and still be capable of acting as intermediate in a rapid equilibration of lipoprotein cholesterol. Experiments to test this hypothesis are now in progress.

Summary. Plasma and red cells containing C^{14}-labeled cholesterol were obtained by feeding dogs with C^{14}-acetate. The labeled blood components were separated and incubated at 37°C for several hours with their unlabeled counterparts under an atmosphere of oxygen-carbon dioxide. The exchange of cholesterol between the two components was followed in each mixture by measurement of the cholesterol specific activities of each component at various time intervals during the incubation. It was observed that equipartition of red cell and plasma unesterified cholesterol was closely approached in 4 hours and 50% equilibration took place in one hour. Esterified cholesterol of plasma did not take part in the exchange phenomenon under these conditions. The possible significance of these results in relation to the nature of lipoproteins is discussed.

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Received July 18, 1951. P.S.E.B.M., 1951, v78.

Complement-Fixing Murine Typhus Antibodies in Vitamin Deficiency States.* II. Pyridoxine, and Nicotinic Acid Deficiencies. (19065)

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The authors have presented data(1) concerning the effects of adequate vit. B complex, 1/10th optimal level vit. B complex, total

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* This investigation was supported by a research grant from The National Institutes of Health, Public Health Service.

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vit. B complex, pantothenic acid and thiamine deficiencies upon complement-fixing antibody production by rats in response to washed murine rickettsiae (*Rickettsia typhi*) as the antigenic stimulus. The present paper deals with the effects of pyridoxine, and nicotinic acid upon complement-fixing antibody production under similar experimental conditions. The purpose of this investigation, and others in progress, is an attempt to find an explanation for the increased susceptibility and mortality that occurs in malnourished populations exposed to typhus fever(2). Wertman and Sarandria(1) reported that pantothenic acid and thiamine deficiencies influenced the antibody response when a relatively small amount of immunizing material was injected. However, the influence was not as apparent when relatively large amounts of the antigenic material were employed. A severe impairment of antibody response has been observed in pyridoxine deficient rats (3,4).

Experimental. Male weanling albino rats of the Sprague-Dawley strain, distributed into groups as indicated in the table, were housed individually in wide-meshed, screen-bottom cages and weighed weekly. The ingredients used in preparing the different diets were obtained from General Biochemicals, Inc. and Nutritional Biochemicals Co. All ingredients were of the highest purity obtainable and each basal diet formulated so that only a minimum amount of the B complex vitamins to be studied were present. The basal diet for the pyridoxine groups was as follows: sucrose, 65.12%; casein (vitamin free), 17.23%; vegetable oil (hydrogenated), 9.55%; salt mixture No. 2, 3.88%; cod liver oil (Squibb), 2.00%; corn oil (Mazola), 2.00%; choline chloride, 0.20%; i-inositol, 0.01%; and paraminobenzoic acid, 0.01%. In preparing the nicotinic acid basal diet, casein was held to a minimum for casein has a high content of tryptophan which is a precursor of nicotinic acid(5). Corn grits were employed because of their low tryptophan content. It was felt necessary to add 1-cystine for corn grits contain only a trace of 1-cystine. The basal diet for the nicotinic acid groups was as follows: corn grits, 40.00%; casein, 9.00%; salt mixture No. 2, 4.00%; choline chloride, 0.20%; i-inositol, 0.03%; l-cystine, 0.15%; corn oil (fortified vit. E), 2.00%; sucrose, 44.62%; and vit. K, 0.001%. In addition two drops of Haliver oil (3,000 U.S.P. of vit. A and 24 U.S.P. of vit. D) were given by dropper to each rat once a week. The rats in the pyridoxine and nicotinic acid groups received their supplemental vitamins in the form of a daily pill. The pyridoxine deficient group had pyridoxine omitted from the pill and the nicotinic acid deficient group had nicotinic acid omitted. Each pill was compounded to supply the following vit.(6); thiamine, 40γ; riboflavin 60 γ; pyridoxine, 50 γ; pantothenic acid, 150 γ; nicotinic acid, 150 γ; biotin, 1 γ; folic acid, 1 γ.

At the end of the 6-week period, each rat was injected intraperitoneally with 0.5 cc (0.0073 mg N)‡ of washed, formalinized suspensions of *Rickettsia typhi* (murine typhus).† One cc inoculations were repeated at 7-day intervals until each rat had received a total of 2.5 cc (0.0365 mg N).‡ During this period, the animals were bled 7 days after each inoculation of murine typhus vaccine to determine if the amount of antigenic stimulus played a role. The complement fixation technic employed in this study was identical to that of Plotz(7), Plotz and Wertman(8), and Wertman(9). Fixation was

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TABLE I. Complement-fixation Titers and Body Weight Change of Rats in Vitamin Deficiency States.

		Complement-fixation titers following		Body wt g	
Diet	No.	1 inj, .0073 mg N	3 inj, .0365 mg N	Init, avg, g	Final avg, g
Nicotinic acid deficient group	41	Neg.	1:640	38	75
	42	1:20	1:128		
	43	”	1:128		
	44	”	1:640		
	45	Neg.	1:128		
	46	1:20	1:128		
Nicotinic acid control group	51	Neg.	1:128	38	126
	52	1:40	1:128		
	53	”	1:128		
	54	”	1:256		
	55	1:10	1:640		
	56	”	1:640		
	57	1:20	1:128		
Pyridoxine deficient group	61	Neg.	1:80	35	96
	62	”	1:320		
	63	”	*		
	64	”	1:320		
	65	”	1:320		
	66	”	1:640		
	67	”	1:160		
	68	”	1:160		
Pyridoxine control group	71	1:40	1:128	40	200
	72	1:20	1:640		
	73	1:40	1:128		
	74	1:10	1:128		
	75	1:40	*		
	76	1:40	1:128		
	77	1:20	1:128		
	78	1:40	1:128		

* Animals died.

allowed to take place for 18 hours at 4 to 6°C. The sensitized cells (0.25 ml sheep cells 3% and 0.25 ml amboceptor containing 3 MHD) were added and the tubes incubated for 30 minutes at 37°C in a waterbath. The tests were read following secondary incubation and only 4+ and 3+ fixations were accepted as end-points. The controls necessary to insure valid results were included in all the titrations and serum dilution tests.

Results. The nicotinic acid and pyridoxine control rats at the end of the 6-week feeding period, all had gained weight, appeared healthy and possessed smooth even coats. The rats fed on the nicotinic acid and pyridoxine deficient diets showed the signs which might be expected to accompany their respective deficiency. The initial average and final average weight of each group are recorded in the table. The nicotinic acid deficient group and the pyridoxine deficient group gained much less weight than their respective control group.

The table demonstrates the end-point titers obtained in the complement fixation test using a washed suspension of murine typhus rickettsiae as the antigen. It appears that nicotinic acid deficiency does not interfere with the production of circulating antibodies when even a relatively small amount of antigenic material was injected. A deficiency of pyridoxine, however, did affect the production of circulating antibodies when a relatively small amount of immunizing material was injected. The animals in this group did not produce detectable complement-fixing antibodies. However, the sera specimens obtained after the administration of 2.5 cc (0.0365 mg N) of vaccine did contain demonstrable concentrations of complement-fixing antibodies. A quantitative difference between the pyridoxine deficient and pyridoxine control groups existed when relatively large amounts of murine rickettsia were injected. The animals reported in this paper were bled prior to immunization and tested by complement-fixation. All of these sera were found to be negative.

Summary. These studies indicate that nicotinic acid deficiency does not appreciably influence the production of circulating complement-fixing antibody when either relatively small or large amounts of antigenic stimulation were employed. Pyridoxine deficiency, however, influenced unfavorably the antibody response when either small or large amounts of the antigen were injected. The single immunizing injection (0.0073 mg N) did not bring forth circulating antibodies that were demonstrable by complement-fixation.

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Role of Sodium Chloride, Water, Kidneys, and Adrenals in the Production of Congestive Edema.*† (19066)

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Evaluation of the respective roles of the adrenals and kidneys in the genesis of edema is dependent on the availability of an animal form and technic whereby an edematous state can be regularly and simply produced. The experiments reported here provide evidence that such an edema can be produced in the rat by ligation of the inferior vena cava, if the animal is allowed free access to 1% NaCl solution postoperatively. This animal preparation has been employed to study the effects of removal of the adrenals and/or kidneys on the development of edema. It is demonstrated that edema does not develop in the nephrectomized rat. However, removal of the adrenals in the nephrectomized rat does result in the development of an edematous state, suggesting that renal retention of sodium and water is not an obligatory factor in congestive edema formation.

Methods. Unfasted male rats of the Long-Evans strain, maintained on this laboratory's Diet XIV, were subjected under brief ether anesthesia to various operative procedures, using clean surgical technics, employing midline ventral abdominal wall incisions. The inferior vena cava was ligated at the caudal margin of the liver, proximal to the right renal vein, with a single silk suture, taking care to include only the vena cava in the ligature. Bilateral nephrectomy was carried out by ligation of the renal pedicles distal to the entrance of the adrenal veins into the renal veins. Bilateral adrenalectomy was carried out by cautery dissection, the glands being removed en bloc with surrounding fat, together with any accessory adrenal cortical nodules visualized. Various sham operations were performed for control purposes. Care was taken to suture doubly all incisions, in order to avoid wound rupture postoperatively as the result of excessive edema formation. After recovery from the brief period of anesthetization, the animals were given access to either tap water or 1% NaCl in tap water for drinking purposes and were allowed free intake of Diet XIV. The animals were weighed postoperatively twice daily. Maximum gains or losses in body weight within the first 24-36 hour period postoperatively were noted and calculated as % change from the onset body weight. The data are presented in Table I.

Results. Ligation of the inferior vena cava

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† Grateful acknowledgement is made to Dr. H. M. Evans of the Institute of Experimental Biology, and to Dr. Choh Hao Li of the Department of Biochemistry, for various kinds of assistance. Appreciation is expressed to Marian Cleeves, Elizabeth Swirnofsky, Roslynn Kenigsberg and Paul Yparraguirre for their cooperative efforts.

TABLE I. Effect of Adrenalectomy and Nephrectomy, Singly and in Combination with Ligation of the Inferior Vena Cava, on Fluid Retention and Edema Formation in the Rat, as Manifested by Changes in Body Wt.*

Operative procedure	No. of rats	Fluid intake	% wt change, gain or (loss)	
			% ± stand. error	Range, %
Controls and sham-operations	13	NaCl	3.9 ± .6	—1 to +8
	5	Water	(6.7 ± .5)	
Ligation of inferior vena cava†	50	NaCl	17.3 ± 2.6	—7 to +72
	16	Water	(.4 ± .7)	
Bilateral nephrectomy	13	NaCl	2 ± 1.3	—4 to +12
Bilateral nephrectomy and ligation of inferior vena cava	12	NaCl	4.6 ± 1.7	—2 to +13
	11	Water	.1 ± .6	—2 to +2
Adrenalectomy and nephrectomy	7	NaCl	(3.2 ± .9)	—7 to +5
Adrenalectomy and ligation of inferior vena cava	13	NaCl	20.2 ± 3.2	+5 to +40
	5	Water	(2 ± 1)	—5 to 0
Bilateral nephrectomy, adrenalectomy and ligation of inferior vena cava	11	NaCl	12.5 ± 4.7	—2 to +46
	5‡	NaCl	27 ± 4.5	+21 to +46
	5	Water	(1 ± 1.4)	—5 to +3

* Operative procedures carried out under ether anaesthetic. Animals were given immediate postoperative access to food and either tap water or a 1% solution of NaCl; edema was measured quantitatively by wt changes which were maximal in 24-36 hr.

† All inferior vena cava ligations were carried out between the caudal edge of the liver and the right renal vein.

‡ Represents 5 animals of the preceding group of 11 rats to show extent of fluid retention at its maximum.

at the caudal edge of the liver did not cause edema in animals given access to water postoperatively. If, however, these animals were given free and voluntary access to 1% NaCl solution for drinking purposes, such treatment resulted in retention in the tissue spaces of a major part of the ingested fluid, resulting in an average body weight gain of 17% within a period of 24-36 hours. The retained fluid manifested itself in the form of a peripheral edema, commencing in the caudal half of the body, and gradually involving the entire body (subcutaneous and loose retroperitoneal and mediastinal tissues, with evidence, in some instances, of slight pleural effusion and/or ascites). Such animals became almost globular in shape, and spent their time in constant drinking, exhibiting a marked polydipsia for the proffered saline solution. Within a period of 3-6 days, however, the animals spontaneously lost their edema fluid.

The availability of this method for the production of an acute and massive edema permitted a study of the effects of renal insufficiency and hypoadrenalism. It was found that ligation of the inferior vena cava in the nephrectomized animal was not an adequate stimulus to cause edema formation, nor did nephrectomy without caval ligation cause the development of this condition. Noteworthy, however, was the development of edema in the adrenalectomized animal with ligated vena cava (average body weight increase of 20%). In a similar manner, removal of the adrenals in the nephrectomized rat with ligated cava caused edema formation (average body weight increase of 27% in the 5 most marked examples). It is to be emphasized that intake of unsalted water did not initiate any of the phenomena noted, nor did sham operations eventuate in edema formation.

Discussion. The edema produced in these experiments by ligation of the inferior vena cava is entirely dependent on the availability and intake of an extra source of NaCl and water. Animals with ligation of the inferior vena cava did not develop edema when given access to water alone. It is well established that an adequate salt intake is necessary before edema formation can occur(1). As noted, the rat with ligated vena cava, when given free access to 1% NaCl, exhibits a marked increase in fluid intake which is coupled with a demonstrated relative failure

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to excrete imposed water loads(2). The combination of increased intake and decreased output result in the formation of a temporary acute massive edema which is lost spontaneously upon recovery of renal excretory function. It is presumed that this is coincident with the development of adequate venous collateral circulatory channels and restoration of more nearly normal hemodynamic and hormonal relationships. It is of interest that removal of the kidneys did not permit the development of edema in the rat with ligated vena cava given free access to saline solution, since the fluid intake in these animals remained at a low level. The suggestion must be made that the kidneys may act, either directly or indirectly, as regulators of thirst and appetite for NaCl. The observation that edema does develop in the nephrectomized animal, if the adrenals are simultaneously removed, suggests a reciprocal relationship between these organs in the regulation of thirst and appetite for NaCl, in the presence of venous congestion. Adrenalectomy, *per se,* is known to cause an increased intake of NaCl (3). The adrenalectomized animal with ligated vena cava, but with intact kidneys, given access to saline develops edema to the same degree as does the non-adrenalectomized rat in the present experiments. It is noteworthy that removal of the adrenals in the nephrectomized rat causes a marked thirst and appetite for NaCl sufficient to overcome the effect of nephrectomy and allow the development of massive edema.

To summarize, if venous congestion is produced in an animal which is allowed free access to saline, edema will develop in the presence or absence of the adrenals, or in the adrenalectomized-nephrectomized animal, but not in the nephrectomized animal. The edema appears to be dependent on the development of thirst and appetite for NaCl solution. The kidney thus appears to play an inhibitory role in this phenomenon which is overweighted by adrenal insufficiency.

The observation that adrenal insufficiency is instrumental in allowing edema formation in the nephrectomized animal assumes significance in interpreting the role of the adrenals and kidneys in various clinical states, such as cardiac congestive failure and the nephrotic syndrome. In this respect, Burch and Ray (4), in a recent report on congestive heart failure, have critically reviewed the nature of the etiological factors operative in edema formation, and have stressed the inferential role played by hormonal factors. They point out that the present hiatus in our understanding of the mechanisms of hormonal activity in congestive failure is, to a large extent, consequent on the lack of suitable experimental methods whereby hormonal relationships can be varied under controlled conditions, with resulting certainty that a condition resembling congestive edema will eventuate. It is felt that the present experimental animal remedies the situation by providing an object in which such hormonal alterations can be evaluated. The edematous state demonstrated in these experiments exhibits the pattern and characteristics of the peripheral edema associated with cardiac congestive failure.

The demonstration that hypoadrenalism facilitates the development of edema in the nephrectomized rat subjected to venous obstruction is likewise of interest, because of the recent reports suggestive of improvement in the congestive failure of cardiac origin (5-8), and in the nephrotic syndrome(9-13), after treatment with adrenocorticotrophic

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hormone (ACTH) or cortisone. Such observations suggest that a relative or absolute hypo- or dysfunction of the secretory activity of the adrenal cortex, or of the responsiveness of peripheral tissues, may exist in these conditions.

The increased intake of NaCl produced by ligation of the inferior vena cava in the rat is reminiscent of the similar chain of events in animals treated with desoxycorticosterone (DOC)(14). Insofar as it is possible, on the basis of the gross observations reported here, to hazard an explanation of the role of the adrenal cortex in the edema of congestive failure, it may be remarked that in this condition, as the result of changes in venous pressure, in electrolyte balance, and in protein and volume regulation, changes which in themselves may not seem grossly exaggerated, that a relative disproportion may occur in the secretion and/or peripheral effects and/or excretory rates of adrenal salt retaining steroids (DOC-like) as contrasted with the 11-17 oxycorticosteroids [see reviews by Burch and Ray(10), Stead(15), and Smith (16).] In this connection, comment must be made on a possible relationship between the diurnal rhythm of congestive failure (nocturnal dyspnea, etc.) and a similar rhythmic cycle in the secretory activity of the adrenal cortex.

Summary. The edema produced in the rat by ligation of the inferior vena cava and voluntary intake of 1% NaCl solution is studied as altered by adrenalectomy and nephrectomy. (1) The obligatory role of NaCl in the formation of edema fluid is reemphasized. (2) The venous congestion produced by ligation of the inferior vena cava is not a sufficient stimulus to cause edema formation in the nephrectomized rat, but does cause edema if the animal is nephrectomized and adrenalectomized. (3) Under these conditions, it is demonstrated that the kidney does not play an obligatory role in the formation of the edematous state in the adrenalectomized rat. (4) The evidence supplied by these experiments is discussed in relationship to the role of the adrenal cortex in conditions exhibiting generalized edema (congestive heart failure, nephrotic syndrome).

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Effect of ACTH Upon Gastric Secretion.* (19067)

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The two principal physiological pathways by which the stomach may be stimulated to secrete hydrochloric acid and pepsin are mediated (a) by way of the vagus nerve and (b) by the release of a hormone, gastrin, produced by the gastric mucosa. Evidence will be presented indicating that the adrenal gland and the adrenal corticoids constitute a third mechanism by which gastric secretion may be stimulated. The vagal phase of secretion is activated by sight, smell, and ingestion of food, insulin induced hypoglycemia, and

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psychic stimuli. Emotional stimuli are expressed under cortical control through the hypothalamus and relayed to the vagal centers. Stimulation or excitation along the course of the fiber tracts from the anterior hypothalamus to the vagal centers may produce gastric erosions, perforations or ulcer accompanied by hypersecretion and hyperchlorhydria(1). Although the gastric response to stress is undoubtedly mediated in part at least through the vagus nerve there is evidence suggesting that an additional hormonal mechanism is involved which may be associated with the general adaptation syndrome. The production of gastric and duodenal ulcerations associated with hemorrhage or perforation is an integral and prominent part of the alarm reaction in response to a number of physical and emotional stress factors, which lead to the release of ACTH by the pituitary gland(2) resulting in the secretion of a variety of adrenal steroid hormones including cortisone and closely related compounds. The hypothalamus which plays a key role in this response to stress may be independent of nervous pathways. Recent studies indicate that in response to emotional and systemic stress the cells of the anterior hypothalamus secrete a humeral substance which stimulates the pituitary to release ACTH(3). Keller has shown, moreover, that section of the vagus nerves still leaves open a pathway over which abnormal or excessive impulses from the hypothalamic centers can exert an effect upon the stomach. After bilateral vagotomy experimental lesions in the hypothalamus and brain stem produced focal hemorrhages in the stomach identical in all respects with those observed with the vagi intact(4).

In an effort to investigate the hormonal role of chronic stress upon the stomach ACTH was administered to human subjects daily for 3-4 weeks to determine its effect upon gastric secretion. The repeated administration of ACTH over a period of time simulates chronic stress since it elicits many of the same responses as those of sustained alarm(5) such as a decrease in the number of circulating eosinophiles in the peripheral blood and an increase in the urinary excretion of 17-ketosteroids.

Methods. ACTH was administered intramuscularly in doses of 100-160 mg daily for a period of 3 to 4 weeks to 6 patients with essentially normal stomachs, one patient with a healed duodenal ulcer and one patient with a gastric ulcer. The basal gastric secretion was obtained by inserting a Levine tube into the stomach in the morning, following a 10 to 12 hour overnight period of fasting, and aspirating the stomach for a period of 60 to 90 minutes by continuous manual suction, following the method of Bloomfield *et al.*(6). The first specimen was discarded and then 3 to 5 consecutive 15-minute aspirations were obtained with constant suction. Consistent and reproducible volumes of gastric secretion were observed during these serial aspirations. The diet was kept constant, and whenever possible the aspirations were performed by the same operator. During the study the fasting patient received nothing by mouth; no belladonna alkaloids or sedatives were administered. The end of the tube was positioned roughly in the lower fundus. The collected juice was immediately placed in an ice bath. Studies were restricted to patients who tolerated intubation well. Basal morning fasting secretions were obtained on at least two occasions before ACTH was administered in order to establish a baseline control. The studies were repeated at 3 to 7 day intervals during the administration of ACTH, and were again carried out several weeks after ACTH had been discontinued in order to establish a level of secretion during the recovery period.

The adrenal response to stimulation was confirmed by eosinophile counts and 17-ketosteroid excretion studies. The volume, consistency, blood, and bile content of each specimen were recorded. The pH was determined

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TABLE I. Effect of ACTH Upon Gastric Acidity. Continuous 15 min serial aspirations for one hr (basal secretion).

		Control period		ACTH, days administered			Recovery period		
Patient	Avg values	1	2	7	14	21	1	2	% increase
A.	15 min vol (cc)	17		20	18				
E.		22	14	27	18	18			
D.		20	22	21	30	31	32	32	
I.		21		20					
N.		21	27		25	27	17		
U.		17	20	32	33	35	30	27	
F.		10	15		24		27		
A.	Free HCl (clinical units)	18		55	30				206
E.		20	32	60	78	70			144
D.		20	15	28	18	73	15	15	265
I.		28		80					186
N.		20	32		59	86	21		169
U.		30	47	48	58	60	45	17	27
F.		0	7		70		0		
A.	Free HCl secreted/hr (mg)	44		162	79				268
E.		65	65	236	204	183			263
D.		59	48	85	79	330	70	48	460
I.		35		234					175
N.		61	126		220	332	51		164
U.		74	136	221	280	300	197	64	121
F.		0	15		242		0		

with a glass electrode and the free hydrochloric acid and total acidity were determined by titration with 10th normal sodium hydroxide, using Topfer's reagent and phenolphthalein as indicators. Pepsin was determined by the method of Bucher, Grossman, and Ivy(7). The 24-hour excretion of uropepsin was determined coincidentally with the basal gastric secretion at frequent intervals during the period of ACTH administration and for approximately one week prior to and following ACTH administration. Uropepsin excretion was determined by a modification of the method described by Bucher, Mirsky, and Anson(8).

Results. The continuous administration of 100-160 mg of ACTH daily for periods of 7-21 days resulted in a significant increase in the basal gastric secretion of hydrochloric acid (Table I), demonstrated by an increase in the average free acidity in clinical units and an increase in the secretion of free hydrochloric acid in mg per hour, accompanied by a decrease in the pH of the gastric juice.

A mean increase of 166% in the clinical units of free acid was observed within 7-21 days of ACTH administration in the basal fasting gastric secretion. The total hourly secretion of free hydrochloric acid (expressed as mg per hour) increased an average of 241% during the administration of the hormone (Table III). In some instances the acidity rose within the first week of ACTH administration but in others the gastric acidity was not affected until 14-21 days of ACTH administration. Upon discontinuation of ACTH the gastric acidity returned to the control level usually within a period of 7 days.

A similarly marked increase in the concentration of gastric juice pepsin was observed in the serial 15-minute basal aspirations during 7-21 days of continuous ACTH administration (Table II). The mean pepsin concentration rose 182% in 5 of the 7 patients studied and the pepsin output per hour increased 169%. After the discontinuation of ACTH the pepsin concentration and output per hour returned to the pre-ACTH control levels. The increase in uropepsin excretion paralleled the

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TABLE II. Effect of ACTH Upon Gastric Pepsin and Urinary Uropepsin.

		Control period		ACTH, No. days administered				
Patient	Avg values	1	2	7	14	21	Recovery period	% increase
A.	Gastric pepsin, units/cc	272		472	779			187
E.		512		581	482	471		
I.		572	639	1500			813	135
D.		150	177	847	577	458	170	390
N.		466	274		997	716	444	114
F.		0	232		431		0	86
U.		341	319	339	264	235	267	
A.	Gastric pepsin, units/hr	18500		37760	56000			202
E.		36800		63000	34800	39400		71
I.		48000	53500	120000			65040	124
D.		12000	15600	71000	69300	57000	21800	354
N.		39200	29600		99700	77500	30000	154
F.		0	13900		41500		0	200
U.		23100	25520	43100	35000	33000	28760	77
A.	Urinary uropepsin, units/24 hr	2507		4416	11234		2100	348
E.		2713		5234	9936	9860	2400	266
I.		4889	4920	10270			4795	109
D.		3900	4450	7400	7300	14000	4500	236
N.		2406	2632		4816	5798	2200	130
F.		3186	3104		5486		2737	74.5

TABLE III. Average Increase in Acid, Pepsin and Uropepsin During ACTH Administration.

Gastric pepsin (units/cc)	182%
(units/hr)	169
Urinary uropepsin (units/24 hr)	194
Free HCl (clinical units)	166
(mg/hr)	241

rise in gastric juice pepsin (Table II). The mean uropepsin increase was 194% compared to an average rise of 182% in pepsin concentration. In general the increase in uropepsin excretion may be demonstrated within 48 to 72 hours of ACTH administration, and precedes the increase in gastric juice pepsin, which usually requires 7-21 days of continuous ACTH administration.

The oral or parenteral administration of 200-250 mg of cortisone daily resulted within 24 to 72 hours in an increase in uropepsin excretion similar to that observed with ACTH. The average increase was 88%.

Discussion. These studies indicate that the stomach is capable of responding to hormonal stimulation by the adrenal cortical steroids, as evidenced by a significant increase in the hydrochloric acid and pepsin content of the gastric juice and a rise in the uropepsin excretion in the urine, following prolonged ACTH administration. The site of action of these hormones upon the gastric glands has not as yet been elucidated. Whether they act upon the vagal nerve endings or whether there is a direct cellular action upon the gland itself or an interrelationship with other hormones such as gastrin remains to be determined. The fact that atropine does not inhibit the effect of ACTH upon uropepsin excretion and that 14-21 days of ACTH administration are necessary to increase the gastric secretion of acid and pepsin suggest a direct hormonal effect rather than vagal mediation. Moreover, the appearance of focal hemorrhages in the stomach following hypothalamic and brain stem lesions in vagotomized animals could result from a hormonal effect through the hypothalamic-pituitary-adrenal-gastric pathway. This mechanism could also explain, in part, the gastroduodenal ulcerations characteristic of the adaptation syndrome.

The effect of ACTH upon the stomach is mediated through the secondary outpouring of adrenal cortical steroids rather than to the direct pharmacological action of ACTH upon the stomach. No gastric response to ACTH can be elicited in patients with adrenal insufficiency but a definite effect can be demonstrated, however, by the administration of cortisone. Both cortisone and ACTH elicit a gastric response in patients with normal adrenal function although the effect of ACTH is more pronounced than that of cortisone.

The uropepsin excretion in the urine reflects the peptic activity of the stomach. It is derived from the secretion of pepsinogen directly into the blood stream by the peptic cells of the stomach. Pepsinogen is then transported to the kidneys and is excreted in the urine as uropepsin(8). That uropepsin has its origin in the stomach is evidenced by the disappearance of the enzyme following total gastrectomy and by its absence in the urine of patients with pernicious anemia(9).

Summary. ACTH administered intramuscularly to normal subjects in doses of 100-160 mg daily for periods of 3-4 weeks produced a marked increase in the basal gastric secretion of hydrochloric acid and pepsin associated with an equally significant rise in the urinary uropepsin excretion. In every instance these values were increased to the levels usually observed in patients with active peptic ulcer. These studies indicate that an endocrine relationship exists between the stomach and the adrenal gland and that a hormonal phase of gastric secretion may be mediated by the adrenal corticoids. The pathway by which chronic emotional and physical stress may affect the stomach by a hypothalamus-pituitary-adrenal-gastric pathway is discussed.

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Pathogenic Properties, and "Vertical"* Transmission of the Mouse Leukemia Agent.† (19068)

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It has been recently observed in this laboratory that "spontaneous" leukemia could be prompted to develop in middle aged mice of a foster nursed C3H subline, designated by the symbol C3H(f), and known to be essentially free from this disease, by inoculating such animals within the first 12 hours after birth with centrifugated extracts prepared from leukemic mice of the Ak line, or with normal Ak embryo cell suspensions. Generalized leukemia developed in some of the inoculated animals after they had reached 8 to 11 months of age(2).

This report deals with the inoculations of the centrifugated leukemic extracts into infant mice 12 hours to 6 days old, with the results of preliminary experiments on the filtration of the leukemic agent, and with the transmission of the leukemic agent from parents to their offspring.

Materials and methods. Centrifugated leukemic extracts. Mice of the Ak line that developed leukemia either spontaneously, or as a result of inoculation with a transplanted strain of Ak leukemia(3), were used as donors

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* Vertical transmission designates the passage of a parasitic agent from one generation to another(1), such as rickettsia through eggs in ticks, mosaic virus through seeds in plants, or mammary carcinoma virus through milk in mice. In contrast, horizontal transmission occurs from one individual to another within the same generation, as in smallpox, typhoid fever, or malaria, through contact, carriers, food, or insects, respectively.

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Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the author are the results of his own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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TABLE I. "Spontaneous" leukemia developing in middle aged mice of the C3H(f) line, following subcutaneous inoculation,* in early infancy, with Ak leukemic, *centrifugated* extracts.

Exp. no.	Age at inoc., days	Sex	No of mice inoc.	No. of mice devel. leuk.	Age when leuk. appeared, mo	No. of mice died without signs of leuk.	Age of mice dying not from leuk., mo	No. of mice in good health at this time	Age of mice in good health at this time, mo
1100	<½	F	1	1	9				
		M	4	3	9,11,17	1	17		
1101	<½	F	1	1	9				
		M	3	2	8,17	1	19		
1106	<½	F	1	1	18				
		M	4	4	11,11,14,18				
1261-A	<½	M	2	1	11	0		1	14
1109-A	<1	F	1	0				1	19
		M	3	2	18,19	1	14		
1361-A	<1†	F	2	2	4,9			0	
		M	2	1	9	1	5	0	
1361-B	<1†	F	1	1	9				
		M	1	1	7				
1361-C	<1†	F	4	4	4,5,8,9				
		M	1	1	8				
1373	<1†	F	5	4	6,9,9,9½			1	9
1261-B	2	M	2	2	14½,15½				
1100-B	3	F	2	0		1	14	1	19
		M	3	0		1	15	2	19
1149	4	F	4	1		0		3	19
		M	1	0				1	19
1109-B	5	F	4	4	18,18,18,19				
		M	1	1	18				
1109-C	6	F	2	2	18,20				
		M	3	2	18,18			1	20
	Total		58	41		6		11	

* 0.1 cc of centrifugated leukemic extract of 20% conc.

† These mice were first inoculated (0.1 cc each) when they were less than 1 day old; the remaining leukemic extract was then placed in refrigerator at 0°C, 24 hr; the same infant mice were then reinoculated with the extract (0.1 cc each); a third inoculation of these mice with the same refrigerated extract (0.1 cc each) was then made after additional 48 hr.

of the leukemic organs. Liver, spleen, mesenteric tumor, and peripheral lymph glands of such mice were cut, and ground, physiological solution of sodium chloride being added to obtain cell suspensions of 20% concentration; these cell suspensions were then centrifugated at 3000 RPM (1400 x g) for 15 minutes at 0°C in an International PR-1 Centrifuge; the supernatant was removed and then again centrifugated for 15 minutes at 3000 RPM. The second supernatant was then immediately used for inoculation. A tuberculin syringe and a 27 gauge needle were used for the injection of the suckling infant mice. *Animals.* Mice of a foster nursed C3H subline(C3H(f)) or, in certain experiments mice of the C3H line, were used for inoculations. The incidence of spontaneous leukemia in our colonies of these mice has been less than 0.07%, only 3 cases of spontaneous leukemia having occurred among the more than 5,000 mice of both sexes during the past 6 years(2), and only 1 case of spontaneous leukemia among more that 1500 males and females of the foster nursed C3H(f) subline.

Experimental. Inoculation of centrifugated leukemic extracts. In our preceding report(2), of the 14 C3H(f) mice inoculated when less than 12 hours old with centrifugated Ak leukemic extracts, 7 developed leukemia at 8 to 11 months of age; later on, however, 5 additional mice of this group also died from leukemia; thus, in these 3 experiments (Exp. No. 1100, 1101 and 1106, Table I) a total of 12 out of 14 mice, inoculated when less than 12 hours old with leukemic extracts, developed "spontaneous" leukemia. The results of experiments on the inoculation of C3H(f)

TABLE II. Results of subcutaneous inoculation of *filtered* Ak leukemic* extracts into suckling infant mice of the C3H(f) line.

Exp. No.	Age at inoc.	Sex	No. of mice inoc.	No. of mice develop. leuk.	Age when leuk. appeared, mo	No. of mice died without signs of leuk.	Age of mice dying not from leuk. mo	No. of mice in good health now	Age of mice in good health now, mo
1533	3 hr	F	2	0				2	7
		M	2	1	6½			1	7
1432	< 7	F	4	2	8½,9			2	9
		M	3	0				3	9
1550	<12	F	2	0				2	7
		M	3	1	7			2	7
1420	<12	M	3	1	6½			2	9
1447	<12	F	4	0				4	9
		M	2	2	8½,9				
764-A	8 days	F	2	1	23	1	14		
		M	1	0		1	14		
764-B	8	F	2	1	27	1	14		
		M	2	0		2	16,18		
	Total		32	9		5		18	

* In Exp. 1420, 1432, 1533 and 1550, the suckling mice were inoculated with a filtered extract prepared from spontaneous Ak leukemia. In exp. 1447, 764-A, and 764-B, the filtered extract was prepared from a transplanted[2] Ak leukemia. In exp. 764-A and 764-B the dose inj. was 0.25 cc for each mouse. In all other experiments infant mice were inj. with 0.1 cc each.

infant mice, when 12 hours to 6 days old, with the centrifugated extracts of Ak mouse leukemia, are summarized in Table I. It is evident from these experiments that when infant, suckling mice of the C3H(f) line were inoculated when less than 1 day old, most of them developed "spontaneous" leukemia after they had reached 7 to 11 months of age; in a few instances, some of these mice that were first inoculated when 1 day old, and were again twice reinoculated to increase the total dose injected, developed leukemia even before they had reached 6 months of age (Exp. 1361 A, B, C, and 1373, Table I). When infant mice 3 to 6 days old were inoculated with the centrifugated leukemic extracts, they developed "spontaneous" leukemia only after they have reached 18 months of age, or not at all (Exp. 1100-B, 1149, 1109-B and 1109-C, Table I).

Experiments are now in progress to determine whether C3H(f) mice that had been inoculated with the centrifugated Ak leukemic extracts at 7 to 21 days of age, will eventually also develop leukemia. No leukemia has yet developed in these groups of mice, although more than 18 months have elapsed since some of them were inoculated.

Inoculation of filtered leukemic extracts. The filtered leukemic extracts were prepared in the following manner: A leukemic cell suspension of 20% concentration, freshly prepared in the usual manner(2), from either spontaneous or transplanted(3) Ak leukemia, was centrifugated at 3,000 RPM for 10 minutes, and the supernatant was then passed through a Seitz (S-1 pad) filter, using a water aspirator, under a vacuum pressure of approximately 20 mm of mercury. The filters, with the mounted pads, had been autoclaved, prior to their use, at 15 lbs, 115°C, for 1½ to 2 hours. Immediately after the filtration, the filters were tested with, and were in all instances found to be impervious to, *E. coli*. The suckling infant mice of the C3H(f) line were then promptly inoculated subcutaneously with the filtered extracts. The results of 7 individual experiments on the injection of the filtered leukemic extracts into suckling C3H(f) mice are summarized in Table II. Of the 9 mice that developed leukemia among the 32 inoculated with the leukemic filtrates, none had any evidence of a local growth at the site of the initial subcutaneous inoculation. In 5 experiments, the filtrates were injected into suckling mice less than 12 hours old. Of the 25 mice injected, 7 developed "spontaneous" leukemia at 6½ to 9 months of age; the remaining 18 mice, however, are still only 7 to 9 months old at this time, and it is possible that some of them may later also develop leukemia. In Exp. 764 A and B, older (8 days) C3H(f) mice were injected, because at the time when this experiment was carried out (September 15, 1948) we did not know that the susceptibility of the C3H(f) mice to inoculation with the Ak leukemic agent decreases so rapidly after the first day of life. Of the 7 inoculated mice, 2 developed leukemia at 23 and 27 months of age respectively (Table II). This delayed development of "spontaneous" leukemia in these mice is similar to that observed in those groups (Table I), where older infant mice were inoculated with centrifugated leukemic extracts.

Transmission of the leukemic agent from parents to offspring. Exp. LV-7. On December 1, 1949, 4 C3H infant mice, less than 36 hours old, were inoculated subcutaneously (0.1 cc each) with *Ak leukemic cell suspension* of 20% concentration. Two of these mice died within 19 days of acute transplanted leukemia; the other 2, male No. 304 and female No. 393, remained in apparently good health; they were mated later, and had 2 litters, one born February 14, 1950, consisting of female No. 395 and male No. 295, and another on March 6, 1950, consisting of females Nos. 446, 447, and 448 and male No. 330. The injected father (male No. 304) died when 14 months old without evidence of leukemia, but the injected mother (female No. 393) developed simultaneously "spontaneous" leukemia and also a mammary carcinoma when 16 months old. Among the offspring, male No. 295 of the first litter, developed spontaneous leukemia at 14 months of age, and female No. 447 of the second litter, also died from leukemia at 16½ months of age; at autopsy, 2 small spontaneous mammary carcinomas were also found in this female; her sister No. 446 also developed leukemia at 18 months of age. Of the remaining offspring, female No. 395 died from mammary carcinoma at 13½ months of age, without signs of leukemia. The 2 others are well at 17 and 18 months of age, respectively. (Fig. 1).

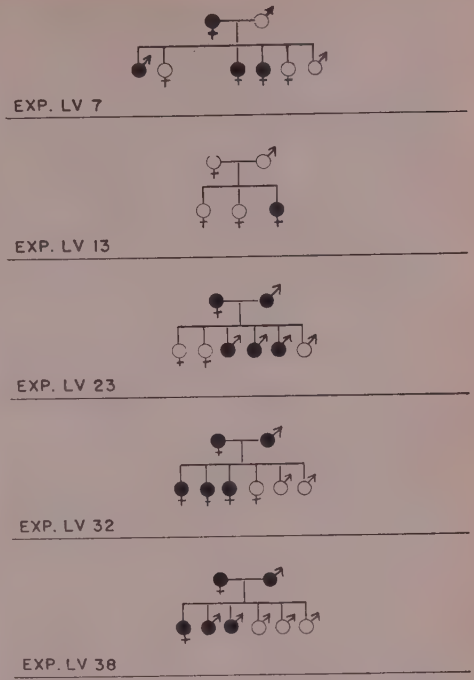

FIG. 1. "Vertical" (from parents to offspring) transmission of the leukemic agent in mice. This figure represents 5 individual experiments designated by their respective symbols. Each experiment consists of 2 generations, parent-mice and their offspring. In all 5 experiments illustrated in this figure, only parents were inoculated (in early infancy) with either centrifugated leukemic extracts (LV 23 and LV 32), with leukemic cell suspensions (LV 7), or with normal Ak-embryo-cell suspensions (LV 13 and LV 38). None of the offspring was treated in any way. Black color indicates the development of "spontaneous" leukemia. It is evident from this figure that 7 of the 10 parents, and 13 of the 27 offspring died from leukemia.

Exp. LV 13. C3H(f) female No. 6 and her brother No. 280 were inoculated, when 4 days old, with *Ak embryo cell suspension* (Exp. 1027) on November 29, 1949. The embryo cell suspension was freshly prepared from normal Ak embryos in the manner described before(2); the pregnant Ak female from which the embryos had been removed by surgical procedure was in good health at the time, but her ancestors died from leukemia. Following the inoculation with the embryo-cell suspension, the C3H(f) female No. 6 and her brother No. 280 remained in good health, were mated, and had a litter on March 3, 1950 consisting of 3 females (Nos. 66, 67, and 68). Both parents died without signs of leukemia at 16 and 20 months of age respectively. Of the 3 offspring, female No. 68 developed leukemia at 20 months of age; the remaining 2 females are now 21 months old, and apparently in good health.

Exp. LV 23. C3H(f) female No. 16 and her brother No. 289 (of the Exp. 1109-C, Table I), were inoculated, along with 3 other litter mates, when 6 days old, with a *centrifugated leukemic extract,* on January 17, 1950. They remained well, at first, were mated, and had a litter on March 22, 1950 consisting of 2 females (Nos. 97 and 98) and 4 males (Nos. 387, 388, 389, and 390). Both parents died from "spontaneous" leukemia at 18 months of age. Some of their offspring, in turn, later on also developed leukemia as follows: Male No. 389 at 16 months, and males Nos. 390 and 388 at 17 and 18 months respectively. The remaining 1 male and 2 females are in good health at 18 months of age.

Exp. LV 32. C3H(f) female No. 93 and her brother No. 65 were inoculated, when less than 12 hours old, together with 3 other litter mates, (Exp. 1106(2), Table I) with *centrifugated Ak leukemic extract* on January 16, 1950. They remained in good health, and had a litter on April 14, 1950, consisting of males Nos. 122 and 123, and females Nos. 136, 137, 138, and 139. Both parents developed "spontaneous" leukemia at 12 and 18 months respectively (Table I). Some of their offspring developed "spontaneous" leukemia, as follows: Female No. 136, at 16 months of age, and 2 weeks later females Nos. 137 and 138. The remaining 2 males and 1 female are well at 17 months of age.

Exp. LV 38. C3H(f) female No. 118 and her brother No. 96 were inoculated, when less than 12 hours old, with *Ak embryo cell suspension* (Exp. 1155)(2); they remained well and had a litter on May 11, 1950 consisting of 1 female (No. 159) and 5 males (Nos. 134, 135, 136, 137, and 138). Both parents died later with leukemia, female No. 118 at 9 months, and male No. 96 at 8½ months of age. Some of their offspring developed leukemia as follows: Female No. 159 at 14 months of age, and males No. 135 and 136 when 15 months old. Male No. 138 died at 16 months without signs of leukemia, and the remaining 2 males are well at 17 months of age. The results of experiments reported in this series suggest that C3H mice which had been inoculated, in early infancy, with the leukemic agent, apparently present in either Ak leukemic cell suspension (Exp. LV 7), in centrifugated leukemic extracts (Exp. LV 23, and LV 32), or in normal Ak embryo cell suspension (Exp. LV 13, and LV 38), will not only, in some instances, eventually develop leukemia themselves, but may, in addition, transmit the leukemic agent to their offspring. In certain instances, either one, or even both, parents may die without symptoms of leukemia (LV 7 and LV 13), but their offspring may nevertheless develop, and die from, this disease.

Discussion. When our first report was submitted for publication(2), only those mice that had been inoculated with centrifugated leukemic extracts when less than 12 hours old, had developed "spontaneous" leukemia; mice that had been inoculated with the centrifugated leukemic extracts at 2 to 7 days of age were still in good health, most of them being over 12 months of age; thus it appeared that the susceptibility of mice of the C3H(f) line to inoculation with the centrifugated Ak leukemic extracts might be limited to their first few hours of life(2). It was observed subsequently, however, that some of those mice that had been inoculated at 1 to 6 days of age, also eventually developed leukemia, although, among those that had been inoculated after their second day of life, the ultimate development of "spontaneous" leukemia was frequently considerably delayed (Table I). Thus, the activation of the leukemic agent in the carrier-host may depend, among other factors, on the time at which the agent was inoculated; the later the agent was inoculated in the life of the infant host, the later its subsequent activation occurred. This was apparent in experiments in which centrifugated leukemic extracts were inoculated (Table I), as well as in those in which the filtered extracts were injected (Table II).

If the trend indicated by the results of experiments on mice injected at 1 to 6 days of age persists also in mice inoculated at older ages, it may well be that most of those injected with the centrifugated leukemic extracts beyond their first or perhaps second week of life, will die their natural death before having had a chance to develop leukemia; the activation of the dormant leukemic agent in most of these mice, may, theoretically at least, occur beyond their normal life-spans, the normal life of a laboratory mouse usually not extending much over 24 months of age.

The results of experiments on the filtration of the leukemic agent thus far obtained suggest that the Ak mouse leukemia agent is filterable through the Seitz (pad S-1) filter. It is true that only 9 out of the 32 inoculated mice developed leukemia; however 7 mice were 8 days old when they were inoculated, *i.e.*, they were injected rather late in infancy, when the susceptibility to the injection with the leukemic agent is greatly diminished, if at all present(2,3); in the second group, 25 infant mice, less than 12 hours old, were used for inoculation, and 7 of them developed leukemia, but these experiments are of a recent date and at least some of the remaining 18 mice, now in good health at only 7 to 9 months of age, may later develop leukemia.

The question may arise whether the development of leukemia in 9 of the 32 mice that were inoculated with the filtered leukemic extracts was a direct result of the inoculation, or whether it actually was spontaneous, and unrelated to the inoculation. It should be kept in mind, however, that spontaneous leukemia is extremely rare, if it occurs at all, in untreated mice of either the C3H line or of the C3H(f) subline. Over 5,000 males and females of the C3H line and more than 1500 mice of the C3H(f) subline have been observed in this laboratory during the past several years, and less than 0.07% of these animals developed spontaneous leukemia. We have also performed a series of experiments in research projects not related to the present study, in which centrifugated extracts, prepared from normal organs of C3H(f) mice, or other non-leukemic extracts (such as centrifugated extracts prepared from fresh, or inactivated mouse mammary carcinoma, from mouse pulmonary adenoma, human breast carcinoma, or serum collected from treated or untreated rabbits) were injected subcutaneously into 1 to 7 days old suckling C3H infant mice(4). Most of the injected mice were then allowed to live their full lifespan, but none of them has developed leukemia. It appears reasonable to state that spontaneous leukemia is most unusual in mice of either C3H or C3H(f) line, and that it cannot be prompted to develop by means of injecting non-leukemic extracts. It would then logically follow, that those mice which developed leukemia in experiments reported in this paper did so because they were injected with the Ak leukemic agent.

The results of experiments dealing with the development of "spontaneous" leukemia in the offspring of mice that had been inoculated with the leukemic agent suggest that the agent, once inoculated into newborn, suckling mice of a hitherto leukemia-free, but susceptible, line, not only infects most of the inoculated animals, causing in them the development of "spontaneous" leukemia, but also passes from the inoculated carrier-hosts into the offspring, which, in turn, may later also develop, and die from, leukemia. This was evident in 5 experiments in which the parents were inoculated with the leukemic agent, but their offspring were not treated in any way; of the 27 untreated offspring born to these parents, 13, or 48% developed "spontaneous" leukemia at 14 to 20 months of age. Even though, however, some of the inoculated parent-mice, particularly those that had been inoculated when several days old, might have themselves remained free from signs of leukemia for the balance of their lives, they apparently carried, and transmitted, the agent in sufficient quantity to cause the development of "spontaneous" leukemia in some of their offspring; this was evident, for instance, in Exp. LV 13, as well as in other experiments(4). Essentially similar observations have been recently reported by Andervont, who found that when serial dilutions of the mammary tumor agent were administered to young mice, an appreciable number of the inoculated animals failed to develop tumors, but their descendants died from mammary carcinomas(5).

There is no reason to believe that the

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3. Gross, L., *Cancer*, 1950, v3, 1073.

4. Gross, L., Unpublished experiments.

leukemic agent would stop at this point its "vertical"*(1,6) trend of transmission; it would be only logical to assume that from this second generation the agent will then pass to the next one, and so forth. Thus, a line of mice hitherto essentially free from this disease, would have changed into a line in which leukemia develops spontaneously in successive generations, as in the case of the leukemic Ak inbred line. A new leukemic inbred line of mice would thus have been originated, at will, by inoculation, in the laboratory. Since, however, the mouse leukemia agent, unlike mouse mammary carcinoma, does not pass from parents to their offspring through mothers' milk(7,8,9,4), but is apparently transmitted directly through the embryos(2), like that of chicken lymphomatosis(10), the investigator would have no means at his disposal of stopping the vertical transmission of mouse leukemia once it has been established in a family of mice.

Summary. 1. When centrifugated extracts of Ak mouse leukemia were inoculated into 16 suckling C3H(f) infant mice less than 12 hours old, "spontaneous" leukemia developed in 8 of them at 8½ to 11 months, and in 5 mice at 14 to 18 months of age. 2. When the centrifugated leukemic extracts were inoculated into 20 C3H(f) infant mice 3 to 6 days old, "spontaneous" leukemia developed in 10 of them, but not before they were 18 months of age. 3. Filtered (Seitz) leukemic extracts were inoculated, in 2 experiments, into 7 C3H(f) infant mice 8 days old, and 2 of them developed leukemia at 23 and 27 months respectively. In 5 additional experiments, leukemic filtrates were inoculated into 25 C3H(f) infant mice less than 12 hours old, and 7 of them developed "spontaneous" leukemia at 6½ to 9 months of age. These results suggest that the Ak mouse leukemia agent is filterable. 4. In 5 experiments, suckling infant C3H or C3H(f) mice were inoculated with either centrifugated leukemic extracts, leukemic cell suspensions, or with normal Ak-embryo-cell suspensions. After the inoculated infant mice have grown up and reached sexual maturity, they were mated, Of the 10 inoculated parents, 7 eventually developed "spontaneous" leukemia at 9 to 19 months of age. Of the 27 untreated offspring, 13, or 48%, have thus far developed "spontaneous" leukemia at 14 to 20 months of age. The results of these experiments suggest that the mouse leukemia agent is transmitted from parents to their offspring. The transmission of the agent occurs most probably directly through the embryos(2,4). 5. The incidence of spontaneous leukemia among the untreated males and females of the C3H or C3H(f) colonies of mice in our laboratory has been, during the past several years, less than 0.07%.

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Mechanisms of Amethopterin Resistance in Leukemia. I. Effects of Weak Folic Acid Antagonists on Mouse Leukemias. (19069)

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In the 20% to 50% of children with acute leukemia whose disease initially responds to amethopterin (4-amino-N^{10}-methyl PGA), the eventual failure of therapy is usually due to the development of resistance to this agent by the leukemic cell(1,2). For this reason experimental studies of the mechanism of this resistance have been undertaken in the hope of finding ways of improving this type of therapy.

In order to conduct such studies, a strain of mouse leukemia resistant to therapy with amethopterin was necessary. A subline resistant to this agent was developed in a previously sensitive lymphoid strain of transplanted leukemia, Ak4(3), by passing the leukemic cells for 4 or more generations through animals treated with amethopterin(4). In the resistant subline, Ak4R(5), there is no difference in the survival time between treated and untreated animals, whereas in the sensitive parent strain, Ak4, the treated animals survive 2 or 3 times as long as the untreated. It is felt that this resistance is a true mutation since there has been no return to sensitivity even after the strain has been passed for more than 30 generations through untreated mice. This line also displays resistance to all other 4-amino derivatives of PGA tested, but not to 2,6-diamino-purine(5,6). The incorporation of radioactive formate into the nucleic acid purines of the viscera of leukemic mice treated with amethopterin is considerably greater with the Ak4R strain of leukemia than with the parent strain(7). Law *et al.*(8) have developed sublines of a lymphoid leukemic tumor resistant to 4-amino-N^{10}-methyl PGA which also display a similar cross resistance to 4-amino-9-methyl PGA, and 4-amino-9,10-dimethyl PGA. One of these strains actually developed dependence and grew better in the mice treated with amethopterin. Weir, Welch, and Heinle(9) showed a definite chemotherapeutic effect from a combination of folic acid free diet, succinyl sulfathiazole, and a crude antagonist of folic acid on a strain of transplanted mouse leukemia in Ak mice. This crude X-methyl folic acid(10) was presumably a mixture of 9-methyl PGA, 7-methyl PGA, and possibly other weak antagonists of folic acid. Although the Ak4R strain is resistant to the 4-amino derivatives of PGA, it was thought possible that it might still be sensitive to antagonists which did not possess an amino group in the 4 position of the pteridine ring.

Method. Mice of the inbred Akm stock were placed on the folic acid free and riboflavin free diet[†] containing 1% sulfasuxidine and a weak folic acid antagonist (either crude X-methyl folic or 9-methyl PGA[†]) for periods of time ranging from 7-13 days before inoculation of the leukemias and were maintained on the diet throughout the course of the experiments. The mice were inoculated intraperitoneally with 0.1 cc of a saline suspension of either Ak4 or Ak4R spleen so diluted that each mouse received 1,000,000 cells. Mice which were not on the special diet, but had been maintained on Purina Laboratory

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[†] We wish to acknowledge at this time the kind generosity of Dr. Gladys Emerson of Merck and Co., for the supply of the folic and riboflavin free diet and to the Calco Laboratories Division and the Lederle Laboratories Division of the American Cyanamid Co. for their supply of the crude X-methyl PGA and 9-methyl PGA.

TABLE I. Survival Time Results.

Exp.		Leukemia AK4 No. mice	Leukemia AK4 Avg surv. time (days)	Leukemia AK4 Inc. surv. time (%)	Leukemia AK4R No. mice	Leukemia AK4R Avg surv. time (days)	Leukemia AK4R Inc. surv. time (%)
I	Control diet				9	13.4	—
	+ ameth @ 2 mg/kg				10	13.2	–1
	Basal diet (no sulfasuxidine)				10	13.4	0
	Basal diet				10	13.9	4
	+ 1% CA				10	16.9	26
	+ 1% 9-methyl-PGA				8	21.9	63
II	Control diet	10	8.2	—	10	10.9	—
	Basal diet + 1% CA	10	13.2	61	9	15.9	46
	+ PGA @ 15 mg/kg				10	12.4	14
III	Control diet	10	9	—	10	11	—
	+ ameth @ 2 mg/kg	8	23.1	157	10	11.6	5
	Basal diet + 1% CA	10	17.8	98	10	18.6	69
	+ PGA @ 30 mg/kg	10	11.6	29	9	13.6	24
	+ riboflavin @ 5 mg/kg	10	17	89	10	17.3	57
IV	Control diet				9	11.8	—
	+ ameth @ 2 mg/kg				9	11.9	1
	Basal diet + .1% 9-methyl-PGA				10	20.2	71
	+ .25% N^{10}-methyl-PGA				10	14	19
	+ 1% CA				10	18.4	56
	+ CF @ 15 mg/kg				10	13.5	14
V	Control diet				10	11.4	—
	+ ameth @ 2 mg/kg				10	12.7	11
	Basal diet + .3% 9-methyl PGA				10	18.6	63
	+ .9% ” ”				7	23.7	108
VI	Control diet	10	9.6	—			
	Basal diet + 1% CA	9	16.2	69			
	+ CF @ .1 mg/kg	9	12.2	27			
	+ PGA @ 7.5 mg/kg	8	7.8	–19			
VII	Control diet	7	8	—	9	13.3	—
	+ ameth @ 2 mg/kg	9	24.1	201	9	13.6	2
	+ PGA @ 7.5 mg/kg	8	17.4	118	10	13.3	0
	+ CF @ .2 mg/kg	9	15.9	99	9	13.8	4
	Basal diet + 1% CA	7	22	175	10	21.1	59
	+ CF @ .2 mg/kg	10	11.9	49	10	15.5	17
	.4 mg/kg	10	9.4	18	10	15.8	19
VIII	Control diet				19	9.4	—
	+ ameth @ 2 mg/kg				10	10.1	7
	Basal diet + 2.5% 9-methyl PGA				10	17.9	90
	+ .00025% ameth				6	9.7	3
	+ .0005% ”				7	9.7	3
IX	Control diet	10	8.8	—			
	+ ameth @ 2 mg/kg	10	14.6	66			
	Basal diet + .000125% ameth	9	14.7	67			

Basal diet = folic acid free and riboflavin free diet plus 1% sulfasuxidine.

Chow, were also inoculated with the same suspension of leukemic cells at the same time to serve as controls. One set of 10 mice, treated with amethopterin, on the regular diet was usually included in each experiment. Intraperitoneal injections of this agent were started 48 hours after transmission of the leukemia at a dosage level of 2 mg/kg, and continued 5 times weekly to a limit of 10 doses. In order to ascertain whether the therapeutic effect of the crude antagonist could be prevented by folic acid (PGA) or citrovorum factor (CF), or riboflavin, these compounds were included in some of the experiments following the same schedule and route as employed with amethopterin. All mice were autopsied at death and if gross manifestations of leukemia were not present, microscopic sections were taken.

Results and discussion. The survival time results obtained with leukemia Ak4 and Ak4R when the mice were maintained on the folic acid free diet supplemented with either the crude antagonist or 9-methyl PGA are presented in Table I. Exp. I in this table shows the lack of effect of the purified diet with or without the addition of the sulfasuxidine when no antagonist was given. There was a definite prolongation of the survival time of mice inoculated with leukemias Ak4 and Ak4R when placed on a combination of the folic acid free diet, sulfasuxidine, and either the crude antagonist of folic acid or 9-methyl PGA. Similar therapeutic effects against strain Ak4R could not be obtained when amethopterin was substituted for the crude antagonist or 9-methyl PGA in this regime. The antileukemic action of the combination of diet, sulfonamide, and crude antagonist could be prevented by giving injections of PGA or CF, but not by riboflavin.

Although the 4-amino derivatives have been shown by Nichol and Welch(14) to prevent the conversion of PGA to CF, the fact that in the mouse and rat a competitive relationship over a wide range has been demonstrated for amethopterin and CF(11-14), but not for amethopterin and PGA, when metabolite and antimetabolite were given simultaneously (15-17), suggests that amethopterin may perhaps be more correctly termed a CF antagonist. The much greater potency of CF than PGA on a dry weight basis in preventing the antileukemic effects of amethopterin also suggests that these therapeutic effects are due to the amethopterin-CF antagonism(18,19).

Ak4R leukemia has been shown to be equally resistant to 4-amino PGA, 4-amino-N^{10}-methyl PGA, 4-amino-9-methyl PGA, 4-amino-9,10-dimethyl PGA, and 4-aminopteroyl aspartic acid(5).

Up to the present time we have been unable to demonstrate any greater content of PGA or CF in Ak4R than in Ak4 spleens, any increased ability of Ak4R spleen suspension to detoxify amethopterin on incubation at 37°C for four hours, or any decreased ability of these same spleen suspensions to adsorb or absorb amethopterin(20). In the light of the above mentioned observations, it would seem that the most likely mechanisms to account for the sensitivity to 9-methyl PGA and resistance to 4-amino-N^{10}-methyl PGA and 4-amino-9-methyl PGA are qualitative changes in the enzyme systems in the leukemic cell for which amethopterin and PGA and amethopterin and CF compete, acting to decrease their relative affinity for amethopterin, or possibly the establishment of an alternate metabolic pathway which by-passes citrovorum factor.

Summary. (1) A definite increase in survival time of mice inoculated with the Ak4 and Ak4R strains of transplantable mouse leukemia was noted when the animals were kept on a purified diet containing no folic acid and no riboflavin, to which had been added sulfasuxidine and 9-methyl PGA. (2) This increase in survival time could be prevented by the administration of citrovorum factor or folic acid, but not of riboflavin.

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Mechanisms of Amethopterin Resistance in Leukemia II. Effect of Cortisone on Sensitive and Resistant Mouse Leukemias. (19070)

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Although cortisone does not cause a prolongation of survival time of mice injected with the Ak4 strain of transplanted leukemia, large single doses will cause a fall in the total leukocyte count in the advanced stages of the disease(1). Since children with acute leukemia may respond well at first but eventually develop resistance either to amethopterin (2-4) or cortisone(5), and since a subline of the Ak4 strain of mouse leukemia has been produced which is resistant to amethopterin (4-amino-N^{10}-methyl PGA), it was thought worthwhile to test the effects of cortisone† on this amethopterin-fast strain (Ak4R)(6) in the hopes that these studies in mice might have some bearing on the problems of clinical resistance to these compounds.

Method. The method used was similar to that previously reported(7). Young Akm mice, 18-22 g in weight, were injected intraperitoneally with 0.1 cc of a saline suspension of spleen from a mouse in the advanced stages of Ak4 or Ak4R leukemia, so diluted as to contain one million cells. Six to 12 days later, when the mice had developed signs of advanced leukemia, and the total leukocyte count had begun to rise, a base line count was taken. Within two hours thereafter a single dose of cortisone was administered subcutaneously. Leukocyte determinations were made at 24, 48, and occasionally 72 hours thereafter.

Results and discussion. From the results with strain Ak4 in Table I it can be seen that a single dose of cortisone at a level of 100-250 mg/kg prevented (temporarily) the precipitous rise in WBC that occurred in the controls, and frequently caused some fall in count. The results in Table II show strain Ak4R to be somewhat more affected by cortisone than its parent amethopterin-sensitive strain. Here single doses of from 25 to 250 mg/kg caused a significant fall in total leukocyte count in 24 hours.

This demonstration of the lack of cross resistance to cortisone in this amethopterin-resistant strain of mouse leukemia is supported by clinical studies(8-10) which show that patients whose disease has developed resistance to aminopterin or amethopterin will still respond to ACTH or cortisone. Clinical studies have also demonstrated that patients whose disease is no longer responding to hormone therapy can often be benefited by treat-

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TABLE I. Effect of Cortisone on Total Leukocyte Count of Mice with Advanced AK4 Leukemia.

Control or treated	Dose, mg/kg	Hr after injection of cortisone					
		0		24		48	
		WBC x 10^3		WBC x 10^3		WBC x 10^3	
		Mean	Range	Mean	Range	Mean	Range
Control	—	86 D	46-157	146 D	62-233	133 A	59-207
Cort.	250	95 E	34-160	36 E	14-68	66 D	23-126
Control	—	49 F	16-95	130 F	30-231	158 C	81-232
Cort.	250	26 G	14-38	42 G	6-72	66 G	9-107
Control	—	18 D	12-27	57 D	33-93	93 B	44-146
Cort.	250	45 F	22-97	38 F	11-67	49 D	39-65
"	125	21	9-41	45	4-95	65 F	6-134
Control	—	29 G	14-49	106 G	29-277	91 B	36-230
Cort.	250	62 E	19-168	66 E	5-140	62 E	11-92
Control	—	20 A	11-28	117 A	75-158		
Cort.	200	49	19-82	34	5-53		
"	100	58	28-91	41	5-83		
"	50	60	12-144	78	36-136		
"	25	45 G	25-74	57 G	10-97		

Ten mice included in average unless otherwise indicated: A-2, B-4, C-5, D-6, E-7, F-8, G-9.

TABLE II. Effect of Cortisone on Total Leukocyte Count of Mice with Advanced AK4R Leukemia.

Control or treated	Dose, mg/kg	Hr after injection of cortisone					
		0		24		48	
		WBC x 10^3		WBC x 10^3		WBC x 10^3	
		Mean	Range	Mean	Range	Mean	Range
Control	—	154 F	119-219	177 F*	107-275*	176 A	148-203
Cort.	250	163	112-205	48 *	14-92*	15 F	7-24
Control	—	76	25-150	79	29-180	71 C	29-154
Cort.	250	73	22-113	29	17-36	35	14-58
"	125	67	20-135	17	6-24	28 G	9-66
"	62.5	59	12-130	25	7-44	21 G	6-51
"	31.0	63	31-116	26	14-42	37 F	21-85
Control	—	76	31-106	76	33-110		
Cort.	200	88	17-129	19	8-31		
"	100	62	36-89	17	8-35		
"	50	77	28-120	20	7-37		
"	25	95	66-138	21	11-28		
Control	—	70	32-119	98	40-141	98 F	58-155
Cort.	125	60	22-97	21	2-38	26 F	8-52
"	62.5	64	17-110	30	11-45	41 G	18-97
Control	—	69 F	26-155	92	29-143	98 D	62-137
Cort.	100	95 G	30-116	47 G	5-66	72 E	29-132
"	50	81	25-113	52	8-74	95 G	69-137
"	25	100 G	42-144	64 G	10-115	85 D	12-133
Control	—	35	18-74	73	50-107	110	41-202
Cort.	31	45	23-85	26	15-34	67 G	25-122
"	15.6	32	17-73	46	15-64	91 G	25-168
"	7.8	30	12-66	62	33-85	94 G	65-158

* Counts taken at 19 hr. Ten mice included in avg unless otherwise indicated: A-2, B-4, C-5, D-6, E-7, F-8, G-9.

ment with the folic acid antagonists(10).

Summary. (1) The administration of large single doses of cortisone to mice with advanced transplanted leukemia of strains Ak4 (amethopterin sensitive) and Ak4R (amethopterin resistant) caused a fall in the elevated leukocyte count. (2) This leukotoxic effect was considerably more marked and occurred

at lower cortisone dosage with the Ak4R than with the parent Ak4 strain, and demonstrates the lack of cross resistance to cortisone in this amethopterin-fast strain (Ak4R) of mouse leukemia.

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Observations on Antiviral Activity of Viscosin.*†   (19071)

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A culture of *Pseudomonas viscosa* was isolated by Kochi in the laboratories of the Yokohama Medical College during a systematic search for microorganisms producing antibiotic substances(1). This culture was found to produce an antibiotic which was active *in vitro* against various pathogenic and saprophytic mycobacteria but which was inactive against representative species of other groups of bacteria. This antibiotic, provisionally designated "P-preparation," was obtained in crystalline form and was found to be heat-stable and soluble in ethanol, methanol, ether, acetone, and alkaline phosphate buffer. Taro Miuri(2) reported that the crystalline antibiotic was found to be an acidic polypeptide having a melting point of 264-268°C (Corr.). In preliminary tests this substance was found to exert a demonstrable therapeutic effect on experimental tuberculosis in guinea pigs(1,3) which was minimal when compared to that of streptomycin(3).

A quantity of the crystalline substance was prepared in this laboratory, and the antibiotic was named viscosin(4). In addition to its antibiotic activity against mycobacteria, viscosin was reported to exert a protective effect in embryonated eggs infected with infectious bronchitis virus of chickens and to exert a slight but detectable suppressive effect on the progress of infection in mice infected with influenza A virus(4). A report of the studies on the antiviral properties of viscosin is presented here.

Methods. The following viruses were used in this study: Vaccinia virus, New York City Board of Health strain; Newcastle disease virus, California strain; influenza A virus, PR-8 strain; influenza B virus, Lee strain; infectious bronchitis virus of chickens, original strain isolated by Beaudette and Hudson; and *Miyagawanella felis,* original strain of feline pneumonitis virus isolated by Baker. Inoculum pools of the various viruses containing 30 to 50% yolk or inactivated normal horse serum were prepared, distributed into suitable ampules, and stored at —70°C. In each experiment the infectivity titers of the various viral inocula were determined in the usual manner by inoculation of serial decimal dilutions of the respective pools into groups of 6-10 eggs each. The contact test, a combined *in vitro* and *in vivo* test was performed as follows: Equal volumes of solutions of various concentrations of the antibiotic and appropriate dilutions of virus were mixed *in vitro* and incubated at room temperature for 2 hours. One ml amounts of the various antibiotic-virus mixtures were then inoculated into groups of 6 to 10 embryonated eggs each. Two *in vivo* embryo tests were used. In the embryo protection test, the antibiotic was injected one hour prior to infection of the embryo. In the embryo therapeutic test, the antibiotic was injected following infection of the embryo as indicated in the text. For vaccinia virus(5) and *Miyagawanella felis* the criterion of antiviral activity was survival of the embryo following inoculation into the yolk sac of eggs in the 6th day of embryonic development. The influenza viruses were inoculated into the allantoic sac of eggs in the 10th day of embryonic development, and the absence or marked reduction of hemagglutinin in allantoic fluid collected from viable embryos 48 hours after inoculation was used as the criterion for antiviral activity. Infectious bronchitis virus and Newcastle disease virus were also inoculated into the allantoic sac but the criterion of antiviral activity was survival of the embryo. All eggs were incubated at 36°C and candled daily through the 16th day of embryonic development. Hemagglutination tests were performed in the usual manner(6).

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‡ It is a pleasure to thank Dr. Selman A. Waksman for encouragement and advice throughout these studies.

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Production of viscosin. The original procedure described by Kochi(1,4) for the production of viscosin was used with minor modifications. The culture medium had the following composition: Glycerol, 20 ml; tryptone (Difco), 5 g; beef extract (Difco), 5 g; yeast extract, 2 g; asparagin, 1 g; KH_2PO_4, 5 g; Mg SO_4, .25 g; distilled water to 1000 ml. The medium was adjusted to pH 7.0 before sterilization in the autoclave. Five hundred ml amounts of medium in Blake bottles were seeded with *Pseudomonas viscosa* 122§ and incubated in the horizontal position at 20°C. Maximum antibiotic activity was obtained after 7 to 10 days' incubation when the pH of the culture was 8.5. The broth at that time contained 100 to 200 dilution units per ml against *Mycobacterium tuberculosis* 607 using the streak dilution method(7). The culture was clarified by centrifugation, the broth adjusted to pH 6, and 2% charcoal (Darco G-60) was added. The mixture was agitated for one hour before filtration. The charcoal was then eluted with a volume of acetone equivalent to 30% of the original volume of broth. The acetone was concentrated under reduced pressure until the viscosin flocculated. Precipitation was completed by storing the concentrated mixture for 1 to 3 days at 4°C. Recrystallization was carried out by dissolving the dried precipitate in 100 times its volume in 50% ethanol at 60°C followed by storage in the refrigerator. The yield of crystalline viscosin thus obtained varied from 200 to 600 mg per liter of culture medium. Solutions of viscosin were made in phosphate buffer at pH 7.3 and sterilized by heating to 100°C for 10 minutes.

Experimental. Viscosin was tested for antiviral activity against 6 viral agents by means of the contact test (see *Methods*). The results are summarized in Table I. It will be seen that infectious bronchitis virus was markedly inhibited or inactivated by viscosin. Slight inhibition or inactivation of the influenza viruses and Newcastle disease virus was also apparent. Viscosin was inactive against vaccinia virus and *Miyagawanella felis* in the concentrations tested. In chicken embryos the maximum tolerated dose (LDo) was approximately 0.5 mg via the allantoic sac and greater than 2.0 mg via the yolk sac. However, the toxicity for eggs varied considerably among groups of embryos infected with the various viruses.

The protective and therapeutic effect of viscosin on infectious bronchitis virus in chicken embryos was determined in the following experiment. Various amounts of viscosin were injected into the allantoic sac of embryonated eggs one hour before, one hour after, and 4 hours after infection, respectively, with approximately 100 LD_{50} of virus. The inoculated eggs were candled daily thereafter and the mortality ratios and the average day of death of the various groups of embryos are presented in Table II. It is clear that injec-

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TABLE I. Antiviral Spectrum of Viscosin in Embryonated Eggs as Determined by the Contact Test.

Virus		Route	M. I. C.* (mg)
Miyagawanella felis	1000 LD_{50}	Y. S.	>2
Vaccinia	100	Y. S.	>2
Newcastle disease	100 ID_{50}	A. S.	.125
Influenza A	100	A. S.	$<$ .063
A	1000	A. S.	.25
B	100	A. S.	.063
B	1000	A. S.	.125
Infectious bronchitis	100000 LD_{50}	A. S.	$<$ .063

Y. S. = yolk sac; A. S. = allantoic sac.

* M. I. C. = Minimal inhibiting concentration: smallest amt of viscosin per ml which exerted a definite suppressive effect.

TABLE II. Protective and Therapeutic Effect of Viscosin on Infectious Bronchitis Virus (IBV) in Chicken Embryos.

1st inj.*	2nd inj.*	Time interval, hr	Result D/T	Result A.D.D.
.125 mg viscosin	100 LD_{50} IBV	1	6/26	
.063		1	8/23	
.031		1	25/26	3.2
Saline		1	29/29	2.2
100 LD_{50} IBV	.125 mg viscosin	1	19/22	4.4
	.063	1	24/25	3.7
	.031	1	20/20	2.8
	Saline	1	41/41	2.4
100 LD_{50} IBV	.125 mg viscosin	4	19/21	3.4
	.063	4	27/27	3.5
	.031	4	18/18	3
	Saline	4	40/40	2.7

D/T = No. embryos dead/total. A.D.D. = Avg day of death.

* .5 ml amt via the allantoic sac.

tion of 0.125 or 0.063 mg of viscosin one hour before infection was highly effective in protecting eggs subsequently infected with 100 LD_{50} of virus. However, when viscosin was injected one hour following infection of the embryos its effectiveness was markedly diminished but not abolished. Treatment of the embryos 4 hours after infection was without significant effect. Attempts to demonstrate a protective or therapeutic effect in embryos infected with the influenza viruses or Newcastle disease virus in embryonated eggs were unsuccessful.

Inasmuch as viscosin protected eggs against 100 LD_{50} of infectious bronchitis virus it was of interest to determine whether there had been a reduction in infective titer. Two groups of 10 eggs each received 0.5 ml amounts of phosphate buffer alone and buffer containing 0.125 mg of viscosin, respectively. One hour later both groups of eggs were infected with approximately 10,000 LD_{50} of virus via the allantoic sac. After 24 hours incubation at 36°C(8) the allantoic fluids from 6 viable eggs of each group, respectively, were collected and pooled. On the same day serial decimal dilutions of each pool were inoculated into groups of 7 eggs each via the allantoic sac, and the eggs were candled daily for 6 days. The infective titer of the pooled allantoic fluids from the control group of eggs was $10^{-7.5}$ whereas the titer of the pooled fluids from the treated embryos was less than $10^{-2.0}$. Thus, it is clear that injection of viscosin into the allantoic sac one hour before infection with infectious bronchitis virus greatly reduced the infective titer of allantoic fluid collected from the treated embryos 24 hours after infection.

Infectious bronchitis virus has been shown

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8. Groupe, V., *J. Bact.*, 1949, v58, 23.

TABLE III. Effect of Auto-interference on Suppressive Effect of Viscosin on Infectious Bronchitis Virus (IBV).

1st inj. (.5 ml)		2nd inj. (.5 ml) viscosin (mg):				
1 part Active IBV	9 parts Heated IBV†	.125	.063	.031	.016	Saline
LD_{50} 1000	Undiluted	2/15*	3/13	3/15	1/13	2/13
”	1/3	5/14	3/15	10/14	9/14	8/13
”	1/9	*7/13*	11/14 D 4.9	12/13 D 3.5	14/14 D 2.8	13/14 D 3.1
”	1/27	*8/15*	12/15 D 5.1	13/13 D 3.3	13/13 D 2.8	11/11 D 2.3
”	Saline	13/14 D 4.6	9/13 D 4.6	14/14 D 2.9	15/15 D 2.6	25/26 D 3

* No. eggs dead/total.

† Undiluted allantoic fluid collected from infected eggs held 24 hr at 36°C after death of the embryo and subsequently heated 75 min at 56°C.

D 3: Avg day of embryo deaths = 3 days after inoculation.

to exhibit the phenomenon of auto-interference when allantoic fluid was collected from infected eggs which were held at 36°C for 24 hours after death of the embryo(8). The influence of the interfering material on the antiviral activity of viscosin is shown in the experiment of Table III. When eggs were infected with active virus suspended in subinhibitory concentrations of heated virus and then injected with inhibitory concentrations of viscosin, the total inhibitory effect clearly exceeded that of viscosin alone. Thus, the therapeutic effect of viscosin on infectious bronchitis virus in embryonated eggs was enhanced when subinhibitory concentrations of interfering material (heated virus) were inoculated together with active virus one hour before treatment.

It was of obvious interest to determine the antiviral activity of viscosin *in vivo* in an adult host. In acute toxicity tests the maximum tolerated dose (LD_0) of viscosin for mice was found to be approximately 50 mg per kg when injected intraperitoneally and greater than 250 mg per kg following subcutaneous injection. Necrosis followed by induration was observed at the site of injection in all mice injected subcutaneously with 50 mg per kg or more of viscosin. Oral toxicity was not determined. A preliminary test on the effect of viscosin on the rate of death of groups of 18 mice each infected intranasally with 100 LD_{50} of influenza A virus showed that daily administration of 1 mg subcutaneously resulted in an average day of death of 5.5 days after infection as compared with 4.5 days in the untreated group. A similar delay in the average day of death of 1.0 day was obtained in another experiment. Since the toxicity of viscosin for infected mice could easily increase with the severity of the disease, the time of appearance and the degree of pulmonary consolidation were utilized as criteria for the measurement of possible antiviral activity in the following experiment. Three groups of 36 mice each were infected with approximately 10,000 ID_{50} of influenza A virus intranasally. One group received no further treatment. Mice in the second group received a single injection of 5 mg of viscosin subcutaneously immediately after infection. Mice in the third group received daily subcutaneous injections of 5 mg of viscosin beginning on the day of infection. Twelve mice from each group were sacrificed on the second, third, and fourth day after infection, respectively, as indicated in Table IV. The lungs of each mouse were carefully examined, and the degree of pulmonary consolidation was noted. The data show that daily administration of 5 mg of viscosin resulted in a slight but detectable reduction in the degree of pulmonary consolidation in mice sacrificed on the 3rd but not on the 4th day after infection. When only one injection of the antibiotic was given, the degree of pulmonary consolidation in the treated mice did not differ appreciably from the controls. Similar results were obtained in each of 6 additional experiments.

Discussion. The antibiotic activity of vis-

TABLE IV. Suppressive Effect of Viscosin on Mice Infected with 10000 ID_{50} of Influenza A Virus.

Viscosin	Mice sacrificed after . . . days	D/T	I/T	Avg score*
0	2	1/12	4/12	.6
5 mg SC × 1	2	0/11	4/11	.4
2	2	0/12	1/12	.1
0	3	4/12	12/12	2.3
5 mg SC × 1	3	3/12	11/12	2
3	3	0/12	8/12	.7
0	4	11/12	12/12	3.9
5 mg SC × 1	4	8/12	12/12	3.3
4	4	8/12	12/12	3.2

SC = subcut. × 1 = 1 inj. immediately after infection. × 3 = 3 daily inj. beginning on day of infection. D/T = No. of mice dead/total. I/T = No. of mice infected/total.

* Score: 1 = 5-25% lung tissue consolidated; 2 = 26-50%; 3 = 51-75%; 4 = 76-100%.

cosin was found to be strikingly specific, not only among the bacteria(1,4) but also among the viruses. The most outstanding characteristic of viscosin observed was its marked protective effect in eggs subsequently infected with infectious bronchitis virus. It is of interest in this connection that subinhibitory amounts of interfering material (heated virus) in the viral inoculum enhanced the suppressive effect of inhibitory amounts of viscosin injected one hour after infection in embryonated eggs. The suppressive effect of viscosin on the progress of infection with influenza A virus in mice was detectable but minimal, and interpretation of the results obtained is difficult. While repeated demonstration of a slight suppressive effect cannot be disregarded, little can be learned from the data save that the effect is minimal. Taken as a whole, the data obtained indicate that viscosin shows little promise of any immediate practical application.

Summary. Viscosin was found to exert a marked protective effect in embryonated eggs subsequently infected with infectious bronchitis virus. A slight but detectable suppressive effect on the progress of infection in mice infected with influenza A virus was demonstrated.

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Adrenal Medullary Stimulating Action of Tetraethylammonium.* (19072)

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The primary action of the tetraethylammonium (TEA) ion is ganglionic blockade. This action results in transitory decreases in blood pressure in most species when the intravenous doses are less than 10 mg/kg(1). Administration to animals of larger amounts of the drug, however, frequently produces rises of blood pressures, although the quantity required to elicit a pressor response appears rather variable(1). In addition, pressor responses have been reported in some human subjects given therapeutic amounts(2), and can seemingly be obtained routinely in patients with pheochromocytoma(3). This hypertensive action of TEA has never been adequately explained. It has been attributed to a direct peripheral constricting effect(4), and more recently, to the release of nor-epinephrine primarily from the liver and to a lesser extent from the adrenals(5). No attempt has been made to explain the pressor action on the basis of ganglionic and/or

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* This investigation was supported by a grant from Parke, Davis Co.

† This research was done during the tenure of a Life Insurance Medical Research Student Fellowship.

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adrenal medullary stimulating properties. Indeed, the possession of ganglionic stimulating properties has been denied(1). Nonetheless, the fact that the ion can cause release of pressor principles from adrenal medullary tumors(3) suggests that the drug may possess an adrenal stimulating property of sufficient intensity to account for the pressor activity observed under the conditions specified. This concept is supported by the fact that pressor effects of the drug are more often obtained, and with smaller doses, in spinal animals(1). Furthermore, weak adrenal medullary stimulating action would not be surprising in view of the close relation between TEA and tetramethylammonium, a well known ganglionic stimulant.

The experiments presented here were performed in an effort to explain more fully the mechanism of the pressor response to TEA, and to gain some insight relating to the rationale for using the drug in diagnosis of pheochromocytoma. A preliminary report of these experiments has been published previously(6).

Method. A preparation first described by Feldberg and Minz(7) seemed suitable for the study of the effect of TEA upon the adrenal medulla. Two to 4 kg cats were etherized, the trachea and jugular vein cannulated, and the cats were then given anesthetic doses of either dial and urethane or pentobarbital, intravenously, or were used in the spinal condition. Evisceration was performed, the renal artery and vein tied close to the hilus of the kidney, and then the inferior vena cava and abdominal aorta were tied just below the origin of the renal artery. Finally, the stump of the coeliac artery was cannulated for retrograde intra-arterial injection into the abdominal aorta. Carotid blood pressure was measured by means of a mercury manometer. Splanchnicotomy or coeliac ganglionectomy was performed on all preparations. The procedure described above drastically restricted the circulation and, if the carotid blood pressure was low (70-100 mm Hg), the preparation served as a sensitive indicator for released pressor principles from the adrenal gland. The sensitivity of this preparation to injected epinephrine appeared to vary inversely with the blood pressure. It was therefore necessary to prevent a high blood pressure due to an overloaded circulatory system in the upper half of the body. This was accomplished by tying the inferior vena cava 1-2 minutes prior to ligation of the abdominal aorta, thus allowing some pooling of blood in the lower extremities. In addition, apparent non-sensitivity of the vacular system to epinephrine was overcome in some cats by using, in addition to blood pressure responses, contractions of the nictitating membrane, made sensitive by removal of the superior cervical sympathetic ganglion 14 to 16 days prior to the experiment. The contractions of the nictitating membrane were recorded on the kymograph by means of an isotonic lever magnifying the responses 12-14 times. The doses of TEA employed are reported herein as total doses and varied from 0.25 to 4 mg. The concentration of drug in saline was adjusted so that any given dose was administered via the arterial cannula in a volume of not more than 0.1 ml, and followed by a saline wash of 0.2 ml over a period of approximately 5 seconds. As a control, frequent injections of saline (0.3 ml) were found to have little or no effect. Comparisons of the effects of TEA administered intravenously with those given intra-arterially were made by a catheter placed in the jugular vein.

The role of the adrenals in the pressor response to retrograde administration of TEA in the coeliac artery was assessed by adrenalectomy after appropriate control responses had been obtained. The adrenalectomy was performed by mass ligation and excision of the whole gland. Test responses to intra-arterial TEA were obtained within 30 minutes of the completion of the adrenalectomy at a time when the blood pressure attained control levels.

Results and discussion. Pressor responses obtainable with relatively large intravenous doses of TEA in intact animals have been reported to vary considerably with respect to

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TABLE I. Effect of Intra-arterial (Abdominal Aorta) Tetraethylammonium on Blood Pressure of Cats.

Preparation	Dose TEA, mg	No. of cats (and injections)		Pressor response, mm Hg ± s
Spinal	4	13	(42)	51 ± 27
Non-spinal	4	6	(22)	41 ± 48

magnitude and even to appearance of a pressor phase(1,5). More consistent results were obtained by Page(5) in spinal animals and in animals with various parts of the autonomic nervous system resected.

A summary of the pressor responses obtained in the present experiments when TEA was administered via the coeliac artery appears in Table I. The results are based on responses obtained with 4 mg, although smaller doses were frequently employed. It is to be noted that much greater variation occurs in non-spinal animals, as evidenced by the large standard deviation. A greater variation in non-spinal animals is not surprising, since concomitant depression of autonomic ganglia would oppose the effect of any released pressor principle. Thus the first dose of TEA administered into the coeliac artery in a non-spinal cat almost always caused a depression of blood pressure, but, at the same time, a contraction of the nictitating membrane. Subsequent doses were almost always pressor, possibly due to the fact that significant ganglionic blockade had occurred following the first dose. Elimination of all the first depressor doses in the data obtained in non-spinal cats in Table I results in a significant reduction in the standard deviation. This value, 55.3 ± 32.6, is more nearly comparable to the values obtained in spinal animals.

In addition to variation in the pressor responses between cats, there was considerable variation to the same dose in a given animal, although this intra-individual variation decreased with repeated administration. This has been reported to occur when TEA is given intravenously to human subjects(2) and to other species(5,8). Variation in the pressor responses to TEA in these experiments in any given spinal animal cannot be explained by various degrees of ganglionic blockade, but may depend on small changes in rate of administration and varying degrees of peripheral sensitization to released epinephrine(9).

It is difficult to compare the doses employed in these experiments with those required to produce pressor responses when the drug is given intravenously. The animals used in these experiments had undergone a drastic reduction in circulatory capacity, and for that reason the doses are reported on a total dose basis. But it is reasonable to assume that a dose of 4 mg intra-arterially in this preparation compares with a 1-3 mg/kg intravenous dose in an intact animal. Such doses usually produce only depressor responses of short duration(1).

The pressor effects of TEA as given in the manner described could conceivably be due to one or more of the following: 1) a direct peripheral vascular stimulating action and/or 2) ganglionic stimulating action involving (a) only the adrenal gland or (b) the adrenal gland as well as other autonomic ganglia. Evidence that a peripheral constricting action plays only a minor role, if any, and that a humoral agent was involved was obtained by comparing the responses of nictitating membrane and blood pressure following intra-arterial (coeliac) and intravenous (jugular) injections of the same dose of TEA. These results are presented in Table II. It is seen that responses of the nictitating membrane and pressor response occur only upon intra-arterial injection. If a direct peripheral constricting action were involved in the pressor response, one would expect greater effects upon intravenous injection than were actually obtained.

Similar comparisons were made with acetyl-

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TABLE II. Comparisons of Effects of Tetraethylammonium Given Intravenously (Jugular) and Intra-arterially (Abdominal Aorta) in the Cat.

			Blood pressure, mm Hg ± s			Nictitating membrane,† mm ± s	
Drug	Dose	Number of cats (and comparisons)	Intra-art.	Intraven.	"P"* value	Intra-art.	Intraven.
TEA (chloride)	4 mg	13 (18)	+48 ± 21	+7 ± 7	<.01	20 ± 12	2 ± 2
Acetylcholine (chloride)	5-10 γ	7 (9)	+27 ± 14	+3 ± 5	<.01		
Histamine (Base)	0.1-2 γ	10 (13)	+22 ± 8	+8 ± 13	<.01		

* Calculated by the "t" test of paired variates. Significantly different if "p" is 0.05 or less.

† Responses are average of 10 comparisons in 6 non-spinal cats.

choline chloride and histamine base; and the results with these agents agree qualitatively with those obtained with TEA (Table II). These results suggested that a pressor substance, probably originating from the adrenals was involved. If such were the case, adrenalectomy should abolish the response, and in addition, furnish additional evidence as to the role of a direct peripheral constricting action in the response.

The participation of the adrenals was proved by adrenalectomy after appropriate control responses had been obtained. It can be seen from the data in Table III that the responses to 4 mg TEA intra-arterially were almost completely abolished following adrenalectomy in each of 6 cats. In addition, the intravenous administration of the adrenergic blocking drugs, piperidinomethyl-3-benzodioxane (933F) and N-ethyl-N-[2-chloroethyl]-9-fluorenamine · HCl (SY-21) in doses which blocked pressor responses to epinephrine likewise abolished the pressor responses to TEA. Blockade of the pressor response to intravenous TEA in intact dogs and cats with adrenergic blocking drugs has been reported(5).

The effects observed after adrenalectomy were slight compared to the control responses. Thus a direct peripheral constricting action of TEA seemed not to be involved, but the released pressor principles from the adrenal gland appeared to account for the entire pressor effect.

Page(5) has recently published evidence which indicates that TEA pressor effects are mediated largely through nor-epinephrine released from the liver. These conclusions were based on the facts that pressor responses to TEA were never reversed, but only blocked, by adrenergic blocking agents, and that adrenalectomy only reduced the response, while hepatectomy completely abolished the response. The results of our experiment indicate clearly that the source of the pressor principle was the adrenal gland; the liver could not be involved in these animals since evisceration and ligation of the coeliac artery removed all effective blood flow to that organ. The results reported at this time neither indicate the nature of the humoral agent responsible nor the reason for the discrepancy between these experiments and those of Page, although it is possible that only the adrenal component was dealt with in these experiments.

TABLE III. Effect of Adrenalectomy on Pressor Responses to Intra-arterial Tetraethylammonium in Cats.

	Blood pressure response (mm Hg)			
	Controls		After adrenalectomy	
Exp.	TEA (4 mg)	Saline (.3 cc)	TEA (4 mg)	Saline (.3 cc)
1	+ 42		+13	0
	+ 43	− 9	+19	
2	+ 94		0	
	+ 88	—	− 9	—
3	+102		+ 8	
	+104	+10	+14	0
4	+102		+18	
	+ 94	0	+ 8	0
5	+ 62	0	+ 4	
	+ 62		+ 2	0
6	+ 36		0	
	+ 36	0	0	0
Mean ± s	72 ± 33		6.5 ± 7.5	

Summary. The injection of .25 to 4 mg of TEA into the blood supplying the adrenal gland of cats with restricted circulation caused definite pressor effects and contraction of the nictitating membrane. The same amounts injected into the jugular vein were without effect. Since adrenalectomy or administration of adrenergic blocking agents abolished the responses, it is concluded that the effects observed were probably due to epinephrine and/or nor-epinephrine released from the adrenal medulla. It is suggested that this simulated nicotinic action is responsible for the pressor effects of TEA that are observed in man and animals under the conditions discussed.

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